ISOLATION OF PATHOGENICITY GENES FROM *XANTHOMONAS* SPECIES AND A STUDY OF THEIR REGULATION

By
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A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

1991

ACKNOWLEDGMENTS

I express my profound gratitude and appreciation to Dr. Dean W. Gabriel, chairman of my supervisory committee for his support, kindness, and sound advice; and to Drs. D.R. Pring, L.C. Hannah and W.B. Gurley, members of my supervisory committee, for their guidance.

I owe special thanks to Dr. R.H. Brlansky for his help with the electron microscopy work. Thanks are also due to Drs. Robert De Feyter and Mark T. Kingsley for their stimulating scientific discussions and delightful company. I also acknowledge the help of Dr. Moosa Hojjati in the maintenance of the plants in the greenhouse facilities. I gratefully acknowledge the technical assistance of Blanca, Susan, and Holly.

I appreciate my friends in Gainesville who were always there in the good times and the bad times. Thanks are also due to my friends Hilisa Bartolome and Leonor Maia, especially for their help during preparation of this manuscript.

I would like to thank my parents and my wife, Nidhi, for their patience during this period of absence from home.

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August 1991

Chairman: Dean W. Gabriel
Major Department: Plant Pathology

A "virulence enhancement" approach was devised for cloning virulence genes from highly virulent pathogens into lesser virulent pathogens, compatible on the same host(s). The approach was to move genomic DNA from a highly virulent disease agent to a less virulent agent and assay transconjugants for increased virulence. Gene *pth*A from the highly virulent pathogen *Xanthomonas citri* was cloned by virulence enhancement of the opportunistic citrus pathogen *X. campestris* pv. *citrumelo*. Transconjugants of *X.c.* pv. *citrumelo* with *pth*A had the ability to induce cankerous lesions on citrus. Marker exchange mutagenesis of *X. citri* revealed that *pth*A was essential for both pathogenicity on citrus and for a non-host hypersensitive response (HR) elicitation on bean. Lack of HR induction by an *X. citri pth*A$^-$ mutant did not extend the host range of *X. citri* to bean

plants. This demonstrated that the avirulence function of *pth*A played no evident role in limiting the host range of *X. citri* on bean and that the hypersensitive response of bean to *X. citri* appeared gratuitous. Gene *pth*A was shown to induce cultivar-specific avirulence when moved into strains of *X.c. malvacearum* (Xcm) and inoculated onto congenic cotton lines. Structural characterization of *pth*A showed that it belonged to a *Xanthomonas avr* gene family consisting of well characterized genes from Xcm and *X.c. vesicatoria*. Gene *pth*A was shown to be 4.5 kb in size and to have 17 nearly identical 102 bp direct DNA repeats in the central region. *In vitro* manipulations of the 102 bp repeats of *pth*A resulted in separation of the pleiotropic functions, pathogenicity and avirulence. Clones were identified which had lost avirulence but possessed enhanced virulence or had lost virulence-enhancement but still expressed avirulence. Three *avr* alleles from *X. citri* and one from *X. phaseoli*, similar to *pth*A, were also found to encode for avirulence in Xcm on several cotton lines. These observations strongly suggest that elicitation of disease symptoms (normosensitive response; NR) and of the disease resistance response (hypersensitive response; HR) may involve very similar but not necessarily the same mechanisms.

CHAPTER I
INTRODUCTION

Plants provide a highly specialized ecological niche for a number of microbial species. Among the many plant- associated bacteria known, those belonging to the genus *Xanthomonas* are always plant-associated and usually pathogenic. *Xanthomonas* as a genus is very widespread, colonizing members of most major plant families. However, individual strains have host ranges restricted to only certain plant groups. This phenomenon of host specialization was once used as the basis for taxonomic speciation of the genus, but this was later revised and all but five *Xanthomonas* spp. were placed into 125 pathogenic variants (pathovars) of one species (Bradbury, 1984). Strains belonging to one pathovar generally are able to attack the same set of plant species and cause similar disease as other members of the same pathovar. However, several pathovars have overlapping host ranges enabling members of different pathovars to be able to attack at least a common subset of host species. Since pathovars have no standing in bacterial taxonomy, suggested standards for their use are not rigorously enforced and often heterogeneous strains will be included in a pathovar based solely on the "host plant from which first isolated" (Starr, 1981) criterion. The main problem with the pathovar system is that the organism is being classified on the basis of a host (response) phenotype (Schroth and Hildebrand, 1983). Recently, phylogenetically-based taxonomies relying on nucleic acid comparisons of strains (Cooksey and Graham, 1989; Gabriel et al., 1989)

are helping resolve the confusion by classifying bacteria on the basis of their genotypes. Once the strains are taxonomically well-defined, the almost exclusive association of certain groups of *Xanthomonas* strains with certain groups of plants and their high levels of host specificity make *Xanthomonas* spp. a model system to study the genetic basis of plant-microbe interactions.

The plant diseases elicited by *Xanthomonas* species are more diverse than the host ranges of the causal agents. The types of symptoms elicited by xanthomonads include leaf spots and streaks (caused by local infection); wilts and blights (caused by systemic or vascular infection); rots (caused by tissue maceration); and cankers (caused by host cell proliferation) (Starr, 1981). Intuitively, altogether different sets of gene functions may be required for the induction of these different kinds of symptoms. Therefore, a comprehensive study of the genetics of virulence of *Xanthomonas* species is likely to involve a number of different experimental strategies appropriate for the kinds of diseases. In this chapter, I attempt to bring forth speculative concepts regarding host specificity and virulence of *Xanthomonas* spp. which have emerged because of some recent work. I also re-emphasize some older concepts which have become better established as a result of recent observations.

Pyramiding of Gene Functions in *Xanthomonas* Species

Strains of *Xanthomonas* spp. are non-obligate biotrophic organisms. This observation has at least three direct implications. *Xanthomonas* strains are chemoorganotrophic and therefore, can be cultured on synthetic media with relative ease.

This indicates that at least a part of the gene functions of these organisms is directed towards their maintenance outside the plant environment and that additional functions are required for host colonization and parasitism (Fig. 1-1). Secondly, being biotrophic, *Xanthomonas* strains do not kill the host cells in advance of their invasion, as necrotrophs do. Genetic mechanisms involved in toxin production are, not surprisingly, relatively uncommon as major or general virulence factors in *Xanthomonas*-caused diseases. Although carboxylic acids from *X. campestris* pv. *malvacearum* and *X.c.* pv. *oryzae* have been reported as blight-inducing toxins, it has been suggested that their phytotoxicity may be solely due to their acidic nature (Robeson and Cook, 1985). The genes involved in conditioning the virulence of biotrophic parasites must be relatively unobtrusive in their effects. The third characteristic of biotrophs like *Xanthomonas* species is that they are well adapted to their hosts and, therefore, have relatively narrow host ranges (Gabriel, 1989). Since these microbes are predominantly plant associated, it is expected that xanthomonads would possess, in addition to conserved general virulence genes, host-specific virulence genes.

All *Xanthomonas* strains appear to be plant associated, as either endophytes or epiphytes. Most but not all naturally occurring endophytic *Xanthomonas* strains are pathogenic but a number of studies have reported isolation of endophytic xanthomonads from asymptomatic tissues. For example, a non-pathogenic endophytic *Xanthomonas* strain capable of multiplying and spreading *in planta* was found to be associated with a number of apple cultivars (Maas et al., 1985). Such strains are difficult to detect in nature because they cause no symptoms. The xanthomonads that have been most studied

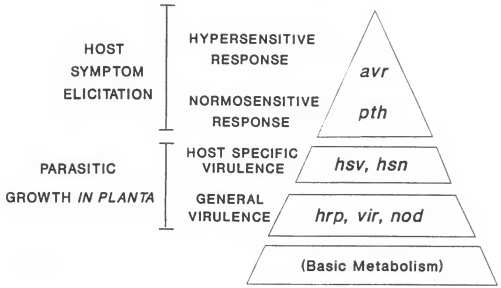


Figure 1-1. Pyramiding of gene functions in *Xanthomonas* spp. Refer to text for explanations of *avr*, *hrp*, *hsn*, *nod*, and *pth*.

are those which were isolated precisely because they caused major detrimental effects. These may be unrepresentative of *Xanthomonas* ecologically. Recently, DNA probes (derived from general virulence genes) have been used to detect opportunistic *Xanthomonas* strains associated with various hosts (Stall and Minsavage, 1990). The ability of non-pathogenic endophytic strains to parasitize (i.e., colonize) host tissue suggests that functions related to pathogenicity--i.e., eliciting a recognizable host response, or normosensitive response (NR) (Klement, 1982)--are superimposed on those required for basic compatibility. Pathogenicity genes add another level in the pyramiding of gene functions in biotrophs, such as *Xanthomonas* spp. (Fig. 1-1). A gene function highly related to pathogenicity may be avirulence; avirulence genes are known to elicit the hypersensitive response (HR), itself thought to be quantitatively but not qualitatively different from the NR (Klement, 1982).

General Virulence Functions

Several general virulence genes have been identified in various *Xanthomonas* species by both chemical (Bonas et al., 1989) and transposon (Kamoun and Kado, 1990) mutagenesis. Mutations in such genes are identified by a loss of the ability of the mutant strain to induce a defense hypersensitive response (HR) in non-host plants and to be pathogenic on the homologous host; such genes are, therefore, termed *hrp* genes (Willis et al., 1991). Hypersensitive reaction refers to the rapid localized plant cell death; characteristic of the interaction when an incompatible pathogen is introduced into plant tissue (Klement, 1982). Strains with HRP^- phenotype are invariably impaired in their

ability to grow *in planta* compared to their wild type counterparts. On the other hand, strains carrying functional *hrp* genes, additionally require expression of specific avirulence genes to induce an HR both on hosts as well as non-hosts (Bonas et al., 1991). Functional *hrp* genes may, therefore, form an essential requirement for HR induction by the pathogen on plants, but these are not the genes responsible for the elicitation of HR.

Sequence and functional similarity of *hrp* genes from strains of *Xanthomonas* and *Pseudomonas* tested to date (Willis et al., 1991) indicates conserved and central role of such genes in determining basic compatibility of Pseudomonadaceae species with plants. All pathogenic *Xanthomonas* strains studied carry *hrp* genes, and opportunistic xanthomonads lack these genes. Information regarding function and regulation of *hrp* genes (organized in clusters on 20-25 kb DNA fragments) (Willis et al., 1991) has mainly been obtained from work in *Pseudomonas* spp. and *Erwinia* spp. An excellent review is available on the subject (Willis et al., 1991). Therefore, it will suffice to mention here that gene *hrpS* from *P. syringae* pv. *phaseolicola* shares sequence similarity with the genes of two-component regulatory proteins and is a positive regulator of at least six other *hrp* genes (Rahme et al., 1991). Additionally, *hrp* genes are expressed at much higher levels *in planta* (Rahme et al., 1991) and in defined minimal medium having low osmolyte concentration *ex planta* (Mindrinos et al., 1990). Functional equivalents of some *hrp* genes from *E. amylovora* are present in *Escherichia coli* and *P. syringae* (Wei and Beer, 1990). It is noteworthy that the doubling time of various pathogenic bacteria *in planta*, compared to doubling time in defined minimal media, seems to be of the same

order. Therefore, *hrp* genes may have evolved from basic prokaryotic functions regulated by nutritional status which are simulated by *in planta* conditions. Over time such regulation could become exclusively associated with genes involved in host-pathogen interaction. This may partly explain the presence of *hrp* gene clusters solely in phytopathogenic bacteria.

Host Specificity Functions

General virulence functions (e.g., *hrp*) genes cannot explain the phenomenon of host specialization of *Xanthomonas* species. Host specific virulence (*hsv*) genes has been used to describe positively acting host specificity genes (Waney et al., 1991). Conceptually, *hsv* genes in *Xanthomonas* spp. may be the equivalent of *Rhizobium* host-specific nodulation (*hsn*) genes. Both *Rhizobium hsn* and *Xanthomonas hsv* genes provide functions which obligatory for colonization and spread in a particular host. The first *Xanthomonas* genes fitting this concept were identified in *X.c.* pv. *translucens* (Mellano and Cooksey, 1988), a pathogen of cereal grains and range grasses. As compared to wild type strains which infected various cereal crops, a number of spontaneous, Tn5, and NTG induced mutants were shown to be restricted on some cereal hosts but not others. Subsequent work has confirmed and expanded these observations, and genes essential for host-specific virulence on wheat and barley have been identified and cloned (Waney et al., 1991). Relative frequencies of Tn5 insertions affecting virulence on one cereal host but not others ranged from 0.11% for rye to 0.04% for wheat, whereas mutations

affecting virulence on all hosts was 0.42% (Waney et al., 1991), suggesting that a larger number of genes are involved in general virulence.

Positively acting genes could impart host-species specificity by either suppressing the active defense response of plants or protecting the bacterial cells from such a defense response in a host specific manner. The *in planta* growth characteristics of host range mutants is a reliable criterion to group the sets of *hsv* genes involved in these two mechanisms. Mutation in defense suppression or defense evasion genes would lead to lethality of the bacterial cells *in planta* (Fig. 1-2). At present, mechanisms enabling a pathogen to actively overcome a host defense response (eg., by detoxification mechanism) are unknown in *Xanthomonas* spp. The detoxification of phytoalexins (host defense related compounds) has been shown to be an important host-specificity determinant in certain fungi (Van Etten et al., 1989). However, there is evidence of mechanisms present in *Xanthomonas* species which may either delay elicitation of development of host defense response or protect the bacterial cells from the defense response once it is elicited.

Could genes involved in exopolysaccharide (EPS) production also be involved in host defense suppression/evasion? Spontaneous and marker-exchange mutations of a locus in *X.c.* pv. *citrumelo* (normally compatible on citrus and bean) have been shown to be lethal in citrus tissue but not in bean (Kingsley and Gabriel, 1991). The affected gene, *hsv*A, confers on *X.c.* pv. *citrumelo* the ability to evade the plant defense mechanisms in a host specific fashion. Mutations at the locus affect EPS production in growth media. Interestingly, isofunctional genes are found in Xcc and *X. phaseoli* which

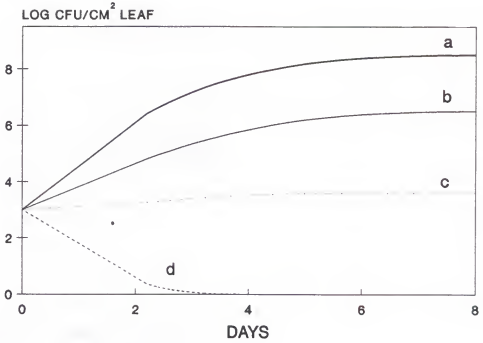


Figure 1-2. Generalized growth characteristics of (a) a virulent strain on a susceptible host; (b) a strain containing an avirulence gene complementary to a resistance gene in the plant or an *hsv*$^-$ mutant strain unable to complement a host-specific function; (c) a heterologous *Xanthomonas* strain; (d) an *hsv*$^-$ strain impaired in ability to evade host defense. The growth characteristics are based on results of experiments involving various host-pathogen combinations. The strains used were *X. citri, X. phaseoli, X.c.* pvs. *citrumelo, malvacearum,* and *translucens*, their relevant mutants and transconjugants. The hosts used were citrus, bean cv. CLR and cotton cv. Ac44 congenic resistant lines.

also restore both HSV function and EPS production in the *X.c.* pv. *citrumelo hsv*⁻ mutant (Kingsley and Gabriel, 1991).

Induction of water-soaking symptoms in leaves infected with *Xanthomonas* species is an indication of a compatible interaction. Purified EPS preparations from *X.c.* pvs. *malvacearum* and *translucens* have been shown to cause persistent water congestion in leaves of susceptible plants but not in those of resistant or non-host plants (El-Banoby and Rudolph, 1979). Therefore, EPS was implicated in host-specificity. Persistence of water-soaking induced by EPS preparation from *X. c.* pv. *malvacearum* has also been shown to be correlated with the virulence (i.e., severity of disease causing ability) of the strain (Borkar and Verma, 1989). However, the authors did not find EPS to be associated with host specificity. Purified EPS preparation from a virulent *X. c. malvacearum* strain induced persistent water-soaking on tobacco, resistant cotton, and cowpea leaves in addition to that on susceptible cotton leaves. It was suggested that EPS production may be negatively regulated in incompatible situations (Borkar and Verma, 1989).

Pathogenicity/Virulence Factors; Cloning Approaches

A number of genes involved in pathogenicity of *Xanthomonas* spp have been isolated. Based on relative frequencies of mutations affecting pathogenicity compared to auxotrophic mutations, it has been estimated that up to 100 genes may be involved in the production of pathogen symptoms (Daniels et al., 1988). This number of genes was found to be comparable with those involved in host-specificity and general virulence of

X.c. pv. *translucens* based on ratio of frequency of such mutations to frequency of auxotrophic mutations (Waney et al., 1991). The experimental approach used could determine the type of genes identified. A widely used approach is to study mutants which are affected in their disease causing ability but not their ability to grow *in planta*. Both chemicals and transposons have been used to induce mutations affecting pathogenicity function (Daniels et al., 1988).

A second approach has been to clone pathogenicity genes specifically induced *in planta*, using reporter gene fusions. For example, a DNA library of ~500 bp fragments from *X.c.* pv. *campestris* (Xcc), a cabbage pathogen, was constructed in a promoter probe plasmid containing a promoterless chloramphenicol acetyl transferase (CAT) gene (Osbourn et al., 1987). Expression of the CAT gene would result from the presence of promoter-active fragments cloned upstream of the gene; this would confer chloramphenicol resistance to the bacterial strain carrying the plasmid. Susceptible cabbage seedlings infiltrated with chloramphenicol were used to select clones containing plant inducible promoters (Osbourn et al., 1987), and a number of such promoters were shown to play a minor role in pathogenicity (Osbourn et al., 1990). In another example, the promoterless *lux* operon cloned in Tn1721 was used to disrupt pathogenicity functions and to create transcriptional fusions and was used to screen for differential expression in plants (Shaw et al., 1988). Using this approach, a gene from Xcc which was highly expressed in the xylem tissue was identified which affected pathogenicity on cabbage. The product of this gene was shown to be exported and the mutant Xcc strain could, therefore, be complemented *in planta* by co-inoculation with the wild type strain. The

gene was later found to be to be functionally *hrp* related and was named *hrp*Xc (Kamoun and Kado, 1990).

As an alternative to the mutagenesis approach to search for host range genes, a "shuttle strategy" was also attempted (Sawczyc et al., 1989). The strategy involved reciprocally transferring DNA libraries of two strains (belonging to *X.c.* pv. *campestris* and *X.c.* pv. *translucens*), having non-overlapping host ranges, and screening the transconjugants on the host of the donor strain. Although the approach was successful in cloning genes involved in the production of extracellular enzymes, no host range genes were identified (Sawczyc et al., 1989).

In any assay when the plant response is relatively slow, stable broad host range vectors are required. Since studies of growth *in planta* are often required, stability of the cloning vectors becomes critical. A series of highly stable, small (8.5-10.5 kb) shuttle vectors for cloning and complementation in a wide range of phylogenetically distinct xanthomonads are now available (DeFeyter et al., 1990; DeFeyter and Gabriel, 1991) and were used for this purpose.

Extracellular Enzymes as Virulence Factors

In most *Xanthomonas* spp. extracellular enzymes do not play a major role in pathogenesis. Mutants affected in the production of a number of such enzymes are known to be as pathogenic as their wild type strains (Daniels et al., 1988). It has been suggested that perhaps their production *ex planta* may aid the *Xanthomonas* strains during their epiphytic phase.

X.c. pv. *campestris* causes black rot of crucifers and a number of cell wall degrading enzymes play a role in its pathogenesis (Daniels et al., 1988). Genes involved in production of amylase, protease (PRT), pectate lyase (PL), endoglucanase (EGL) and polygalacturonase (PGL) have been isolated from Xcc (Daniels et al., 1988). Marker exchange mutagenesis has shown that mutant Xcc strains affected in protease encoding (*prt*) genes (Tang et al., 1987) and endoglucanase-encoding (*egl*) genes (Gough et al., 1988) are only slightly impaired in their pathogenic functions. However, Tn5 mutations in a 10 kb region of Xcc genome containing genes for export of cell wall degrading enzymes lead to a complete loss of pathogenicity (Dow et al., 1987). An export-related gene *xex*A from Xcc present in 5.3 kb of this cluster contains three open reading frames (ORFs), two of which appear to code for transmembrane proteins and one has homology to the *vir*B orf11 of *Agrobacterium tumefaciens* (Dums and Daniels, 1990). Sequences similar to extracellular enzyme export related genes of Xcc are present in other *Xanthomonas* species, although their significance in pathogenicity of other pathovars is as yet unknown (Dums and Daniels, 1990).

Exopolysaccharides as Virulence Factors

Exopolysaccharides provide a hydrophilic surface for the bacterial cells and thereby facilitate absorption and binding of water molecules. Intercellular spaces in the leaf mesophyll are initially air-filled; with water congestion they become ideal for bacterial multiplication. It has been calculated for the related genus *Pseudomonas syringae phaseolicola* that there are sufficient nutrients per plant cell to support 60

bacterial cells (Hancock and Huisman, 1981). Along with nutrient availability from the immediately surrounding host cells, diffusion from nearby cells can also take place which can ultimately allow bacterial numbers to increase to as high as 10^8 cfu/cm^2 leaf (Fig. 1-2).

A number of clusters of genes involved in xanthan gum (exopolysaccharide) production have been identified using transposon mutagenesis. An excellent review of the genetics and biochemistry of xanthan synthesis is available (Coplin and Cook, 1990). In brief, two kinds of gene clusters are involved in EPS production in different *Xcc* strains. In one case, a 16 kb cluster containing 12 ORFs (*gum*B-M) transcribed from a single promoter carries out all the required functions. In another strain, a cluster of 12 complementation groups is required. Not all genes in this cluster are transcribed in the same direction.

As in the case of host specificity, reports regarding effects of mutation in EPS related genes or virulence are conflicting (Coplin and Cook, 1990). A positive correlation was established between virulence of Xcc on cabbage and parameters such as final viscosity of the culture, the viscosifying capacity of the polymer and the amount of acetyl substituents in the gum. Intramolecular interaction of gum constituents were postulated to play a significant role in virulence. However, in another study none of the EPS$^-$ mutants were found to be affected in virulence (Coplin and Cook, 1990). It is noteworthy to mention that inoculation technique in the first study involved inoculation via hydathodes, whereas the second one used stem injury for inoculation. Recently, it was shown that some Xcc mutants affected in genes clustered in the 12 complementation

groups displayed reduced virulence in cabbage (Hotte et al., 1990). As noted above, the host-specific virulence locus *hsv*A of *X.c.* pv. *citrumelo* may be involved in EPS production (Kingsley and Gabriel, 1991).

Regulation of Pathogenicity and EPS Related Genes in Xcc

Work on Xcc has shown that genes in a 10 kb pathogenicity cluster and in a 16 kb EPS cluster are both coordinately under positive as well as negative regulation (Daniels et al., 1989). Five regulatory genes (*A-E*) having coordinate effects on production of several extracellular enzymes, exopolysaccharides and pathogenicity have been defined. The regulatory gene *C* has both the "sensor" and "effector" domains combined into one predicted protein sequence. There also exists a parallel system which negatively regulates the synthesis of all three factors in a coordinate manner (Daniels et al., 1989). This was evidenced by the effect of copy number and mutation of the negative regulatory gene on extracellular enzyme production, EPS production and pathogenicity. Exopolysaccharide production seems to be under an additional positive regulatory control system from that of the enzymes and of pathogenicity genes studied (Daniels et al., 1989). Oligonucleotide probes matching postulated coding domains of sensor and regulator proteins, respectively, were used to identify similar sequences in Xcc. Strains of Xcc with Tn5-induced mutations in a putative regulator gene were affected in EPS production but not that of macerating enzymes or pathogenicity.

As has been pointed out by Coplin and Cook (1990), the "demand theory" of gene regulation (Savageau, 1983 cf. Coplin and Cook, 1990) predicts that in bacteria which

alternate between very different niches, pathways will show both positive and negative regulation. In shifting from one niche to another, the predominant mode of regulation may shift. It will be interesting to learn the nature of signals (one being plant specific) which induce the two known positive regulators in Xcc.

Avirulence Genes; their Gratuitous Function and Minor Role in Restricting Host Range at Host Species Level

A distinct class of genes termed avirulence (*avr*) genes have been identified based on their ability to confer host-cultivar specificity to pathogens. Functional avirulence appears to be superimposed on other kinds of gene functions in *Xanthomonas* species (Fig. 1-1). Specific avirulence genes can induce the host defense response and render an otherwise compatible plant-microbe interaction incompatible. Several physiological interpretations of how *avr* genes induce defense responses were recently reviewed (Gabriel and Rolfe, 1990). The biological significance of *avr* genes has long been debated (Gabriel, 1989). When strains carrying *avr* genes interact with plants carrying corresponding resistance (*R*) genes, incompatibility results and the avirulent strains fail to multiply to high numbers (Fig. 1-2). When inoculated with avirulent races of *X.c.* pv. *malvacearum*, resistant cotton lines develop localized zones of inhibition (in ca. 20-40 palisade cell width radius) which leads to restricted bacterial growth (Essenberg et al., 1979).

When mixtures of strains carrying different *avr* genes are coinoculated on susceptible cultivars, the frequency of some *avr* genes rises in the pathogen population. The question has always been raised as to the selective value of *avr* genes for the

pathogen. In the absence of particular resistance genes in plants, why are *avr* genes not lost from the pathogen population? Many *avr* genes do not evidently contribute to fitness of the microbe. Despite much effort, no evidence of stabilizing selection has been documented (Gabriel, 1989). Several *avr* genes are known which do not have any apparent function nor even alleles in other strains of the same taxonomic group. Marker-exchange mutagenesis of most *avr* genes has no detectable effect, except loss of avirulence. A case from *Xanthomonas* spp. is documented where selective value for an *avr* gene has been shown or suggested. Gene *avr*Bs2 of *X.c.* pv. *vesicatoria* directly contributes to pathogen fitness (growth *in planta*) and is fairly widespread in *Xanthomonas* spp. (Kearney and Staskawicz, 1990).

It has been argued that avirulence genes may act at higher specificity levels and be responsible for host range determination at these levels (Keen and Staskawicz, 1988). However, elimination of the non-host HR is not known to result in an expansion of host range beyond that already described for the pathogen. Although inactivation of *avr*BsT of an *X.c. vesicatoria* pepper race allows it to attack tomato plants (Minsavage et. al., 1990), tomato is a known host of *X.c. vesicatoria*. The frequency of this race change mutation is 10^{-4}/generation (Dahlbeck and Stall, 1979). Similarly, inactivation of *avr*BsP of *X.c.* pv. *vesicatoria* allows strains of a tomato race to become pathogenic to pepper (Canteros, 1990), but pepper is also a normal host for *X.c. vesicatoria*. These two *X.c. vesicatoria avr* genes are involved in race specificity, superimposed on an existing ability to parasitize both tomato and pepper. Other than race change mutations of strains with

known host range, there is no evidence to support the idea that avirulence genes limit the host range of pathogens above the race level.

Several Members of a *Xanthomonas avr* Gene Family Pleiotropically Affect Normosensitive Response

Evidence for a *Xanthomonas avr* gene family was first provided by hybridization data of many pathovars of *Xanthomonas* spp. using *avr*Bs3 of *X.c. vesicatoria* as probe DNA (Bonas et al., 1989). Subsequently, a group of six *avr* genes from *X.c. malvacearum* were demonstrated to be similar to each other and to *avr*Bs3 (DeFeyter and Gabriel, 1991). Another gene, *avr*BsP of *X.c. vesicatoria* also belongs to this gene family (Canteros, 1990). The members of the family are frequently plasmid borne and unusually large sized (3-5 kb) for prokaryotes. The large size of the genes is due to the presence of nearly identical 102 bp direct DNA repeats in the central region (Bonas et al., 1989).

It has been previously suggested by Klement (1982) that the difference in the two kinds of host responses, viz., normosensitive and hypersensitive (Fig. 1-1) is because of the timing of these responses and that they are distinguishable by the relative numbers of bacterial cells required to elicit each type of response. It has been observed that while in an incompatible interaction 1:1 ratio of bacterial to host plant cells (with relative volume ratio of $1\mu m^3$ to $5X10\mu m^3$) can lead to HR development, as many as 50 bacterial cells per host cell are required to induce the same host responses in a compatible interaction (Klement, 1982). Studies with *Arabidopsis* defense related genes, *pal* and *chs*, involved in the production of phenylammonia lyase and chalcone synthase,

respectively, in reponse to Xcc inoculations also show that although host defense is triggered in both compatible and incompatible interactions, the induction kinetics of these genes are slower in a normosensitive reaction (NR) compared to those in a hypersensitive response (HR).

Two members of the *Xanthomonas avr* gene family have been shown to induce both the NR and HR in plants. The genes, *avr*b6 and *avr*b7 of *X.c.* pv. *malvacearum* affect the water soaking ability in compatible interactions (DeFeyter and Gabriel, 1991). This suggests that at least some *avr* genes may be triggering both kinds of host reposnses and that these "triggers" for both normosensitive and hypersensitive responses may be very similar in the host plants.

CHAPTER II
A PATHOGENICITY LOCUS FROM *XANTHOMONAS CITRI* ENABLES STRAINS FROM SEVERAL PATHOVARS OF *X. CAMPESTRIS* TO FORM CANKER-LIKE LESIONS ON CITRUS

Introduction

Xanthomonas citri (ex Hasse) nom. rev. (Gabriel et al., 1989) is the causal agent of Asiatic citrus canker disease. The host range of the pathogen includes a wide variety of *Citrus* spp. and relatives in the Rutaceae. Untreated infestations can result in defoliation and premature fruit drop leading to serious economic losses (Schoulties et al., 1987). Symptoms of Asiatic citrus canker disease include erumpent, corky lesions on all aerial parts of mature citrus trees including fruits, leaves and stems (Schoulties et al., 1987). *X. campestris* pv. *citrumelo* pv. nov. Gabriel (Gabriel et al., 1989) is the causal agent of citrus bacterial spot. These pathogens produce only mild or opportunistic leaf spot infections on juvenile citrus foliage (Graham and Gottwald, 1990). *X.c.* pv. *citrumelo* strains are a heterogeneous group, genetically and pathologically related to other heterogeneous pathovars, including *X.c.* pv. *alfalfae* (Gabriel et al., 1988), *X.c.* pv. *cyamopsidis* (Gabriel et al., 1989), *X.c.* pv. *fici* and *X.c.* pv. *maculifoliigardeniae* (Graham et al., 1990).

Various approaches to cloning pathogenicity determinants in *Xanthomonas* spp. have been employed, including rapid *in vitro* plate assays (Tang et al., 1987) and

complementation of mutations affecting pathogenicity using *in planta* assays (Turner et al., 1985). Another successful method involves identifying plant-inducible promoters in "promoter-probe" vectors, with subsequent identification of any pathogenicity genes that may be transcribed from the promoters (Osbourn et al., 1990a). A strategy for cloning virulence genes that has been attempted without success was that of trying to increase the host range of a strain from one *Xanthomonas* pathovar by adding DNA fragments from a strain of a different pathovar having a non-overlapping host range (Sawczyc et al., 1989). A strategy similar to the latter approach might work, if the recipient strain was at least mildly compatible with the host plant, and if there were easily scorable differences in virulence or disease phenotypes. The fact that citrus can serve as a host for both a *Xanthomonas* species that causes severe disease and one that causes only mild or opportunistic infections led us to attempt the "virulence enhancement" approach. The approach is based on the hypothesis that the disease phenotype induced by a pathogen like *X. citri* can be attributed to gene functions that induce pathogenic reactions in plants (ie., pathogenicity genes), in addition to those required for growth *in planta* (ie., parasitism genes) (Gabriel, 1986). Such pathogenicity genes from highly virulent strains should be either absent or non-functional in milder pathogens compatible on the same host.

The cloning of a DNA fragment carrying a pathogenicity (*pth)* gene locus that is required by *X. citri* to induce the plant symptoms associated with Asiatic citrus canker is described in this chapter. The locus was identified by screening an *X. citri* library in *X.c.* pv. *citrumelo* for virulence enhancement of the milder pathogen on citrus.

Materials and Methods

Bacterial Strains, Plasmids and Culture Media

Strains of *Escherichia coli*, *Xanthomonas* spp., and plasmids used in this study are listed in Table 2-1 along with their relevant characteristics and source or reference. *Xanthomonas* spp. were cultured on PYGM medium at 30C, as described previously (Gabriel et al., 1989). *E. coli* strains, unless otherwise stated, were grown in LB medium at 37°C (Miller, 1972). Antibiotics were used at the following final concentrations (in μg/ml): Chloramphenicol (Cm), 35; Kanamycin (Km), 25; Nalidixic acid (Nal), 100; Spectinomycin (Sp), 35; Streptomycin (Sm), 100; Tetracycline (Tc), 15; Gentamicin (Gm), 1.5 for *E. coli* and 3 for *Xanthomonas* spp.

Genetic and Bacteriological Techniques

Xanthomonas citri 3213^T genomic DNA, partially digested with *Sau*3AI, was size-fractionated on a sucrose density gradient and the 20-25 kb fraction ligated to *Bam*HI linearized pUFR027 DNA. Competent *E. coli* DH5α cells were transformed and plated on selective medium containing Bluo-gal (halogenated indolyl-ß-D-galactoside; Bethesda Research Laboratories., Gaithersburg, MD) and IPTG (isopropylthio-ß-galactoside; Bethesda Research Laboratories) according to Sambrook et al. (1989). Eighteen white colonies from the clone bank were randomly chosen, their plasmid DNAs were extracted by alkaline lysis (Birnboim and Doly, 1979), and the restriction profiles of each were

analyzed to determine the average insert size. The *X. citri* 3213 clone bank was introduced into *X. c.* pv. *citrumelo* 3048 using *E. coli* HB101 (pRK2013) as a helper strain in triparental matings (Ditta et al., 1980). Unless otherwise stated, the same conjugation method was used to transfer other clones into various *Xanthomonas* strains used in the study.

Standard recombinant DNA procedures (Sambrook et al., 1989) were followed for various cloning and restriction mapping experiments. Southern blot analyses (Southern, 1975) were performed using Genescreen Plus nylon membranes (NEN Research Products, Dupont, Boston, MA) according to the manufacturer's recommendations. The membranes were treated and probed with ^{32}P-dCTP radiolabelled plasmid DNA as described previously (Lazo et al., 1987). For gene expression studies using the Tn*5-gus*A promoter/probe transposon (Sharma and Signer 1990), ß-glucuronidase assays were performed as described by Jefferson (1987).

Fragments from pUFR027 (Nm^r) were subcloned in pUFR042 (Nm^{r}Gm^r) or in pUFR044 (Gm^r). pUFR049 (Cm^{r}Sm^r) was constructed by inserting a 1.0 kb *inc*W fragment from pUFR034 (DeFeyter et al., 1990) into an RSF1010-derived replicon.

Transposon Mutagenesis

Transposon mutagenesis was carried out in a three step process. The target clone (p35KX15) (Gm^r) was used to transform competent *E. coli* C600-387 cells, which harbors the transposon Tn*5-gus*A on the chromosome. Tn*5-gus*A produces

transcriptional fusions (Sharma and Signer, 1990) on insertion in one orientation in a transcriptionally active gene.

Approximately 250 independent transformants, resistant to Km, Tc, and Gm served as donors in triparental matings using *E. coli* HB101/pUFR035 (Sm^rCm^r) as the recipient and ED8767/pRK2073 (Sp^r) as the helper strain. Mid-log phase cultures of the recipient and helper strains (mixed 1:1) were spot-inoculated (10 to 20 μl/spot) on LB agar plates. After the excess liquid was absorbed by the agar, transformants (donors) were transferred onto and mixed with the individual spots of recipient and helper using sterile toothpicks. After incubation of these patch mating plates at 37C for 4-6 hours, cells from each patch were transferred to LB plates containing Sm, Cm, Km, Tc and Gm using sterile toothpicks [i.e. HB101/(pUFR035 + p35KX15::Tn*5-gus*A)].

In the final step, pUFR035 was eliminated by conjugally transferring p35KX15:: Tn*5-gus*A derivatives into an *E. coli* strain (C2110 Nal^r) carrying a *pol*A mutation. Recipient *E. coli* C2110 and helper ED8767/pRK2073 strains were spot-inoculated and donor cells subsequently patched on them as before. Transconjugants were selected on plates containing Nal, Km, Tc, and Gm. Two colonies were retained per each initial mating, making a total of ~500 Tn*5-gus*A insertional derivatives. The site and orientation of the inserted transposons were determined by restriction analyses.

The mutagenized clones were introduced into various *Xanthomonas* strains (Sp^r) by triparental matings using individual *E. coli* C2110/p35KX15:: Tn*5-gus*A ($Nal^rKm^rTc^rGm^r$) clones as donors and HB101/pRK2013 (Km^r) as the helper strain. Conjugations were harvested on selective plates containing Sp, Km, Tc and Gm.

Marker-Exchange Mutagenesis

Marker-exchange mutagenesis of wild type *X. citri* 3213 (Sp^r) was accomplished by mobilizing the IncW displacement vector pUFR049 (Cm^rSm^r) with pRK2013 into transconjugants harboring p35KX15::Tn*5-gus*A ($Gm^rKm^rTc^r$) derivatives. Transconjugants were selected on plates containing Sp, Km, Tc and Cm. Approximately 50 transconjugants from each mating were screened for Gm^s. The $Sp^rKm^rTc^rGm^s$ clones were then grown on medium lacking Cm to allow for segregation and loss of pUFR049, and Cm^s colonies retained. These marker-exchanged mutants were maintained on plates containing Sp, Km and Tc.

Pathogenicity Screening and Testing

All plants were grown under natural light in quarantine greenhouse facilities equipped with HEPA air filters at the Division of Plant Industry, Florida Department of Agriculture, Gainesville, FL. Temperatures in these greenhouses normally range from 28ºC to 35ºC, and from 50% to 100% relative humidity. All inoculations were carried out under BL-3P level containment in these quarantine greenhouses (refer Federal Register Vol. 52, No. 154, pp 29800-29814, August 11, 1987).

Over 500 transconjugant *X.c.* pv. *citrumelo* 3048 strains were screened in duplicate inoculations for virulence enhancement on *C. paradisi* cv. "Duncan." Transconjugants were picked from PYGM agar plates which had been incubated at 30C for approximately 60 hrs. Colonies were resuspended in sterile tap water, and inoculated through the abaxial surface of immature Duncan grapefruit leaves by pressure infiltration

using blunt ended syringes (Gabriel et al., 1989). Although bacterial concentrations in the inocula were not determined in these preliminary screenings, care was taken to pick approximately the same amount of cells from the PYGM agar plates. Symptoms were recorded 8-12 days later.

In retests of putative virulence-enhancing clones, transconjugants and control strains were grown to late log phase, and the concentrations of all inocula were adjusted to 10^8 cfu/ml using a spectrophotometer. Grapefruit leaves were inoculated as before. Inoculations of fully expanded, mature leaves of *Cyamopsis tetragonoloba* USDA PI 215590 cv. 13643 and *Phaseolus vulgaris* cv. California Light Red (Agway Corporation Beanplant, N.Y.) were performed by pressure infiltration as described previously (Gabriel et al., 1989).

In planta Growth Kinetics

Bacterial suspensions, adjusted to $\sim 10^5$ cfu/ml in sterile tap water, were pressure-infiltrated into fully expanded, yet immature, *C. paradisi* cv. "Duncan" (grapefruit) leaves. These leaves were all of similar size and thickness. Leaf tissue disc punches (8 mm diameter) were removed at 0, 1, 5, 10 and 15 days after inoculation from non-wounded leaf zones, macerated in 1 ml sterile tap water, and appropriate dilutions plated to obtain single colonies on media containing appropriate antibiotics. Growth of *X. citri* 3213, B21.2, and B21.2/pSS35KBg *in planta* was determined by counting only those colonies which carried all appropriate antibiotic markers. Populations were expressed as log cfu/ml of sterile tap water used for grinding leaf tissue.

Stability of plasmids *in planta* was determined by plating samples from the above leaf extracts on PYGM (Sp) plates. One hundred randomly chosen colonies from each time point were screened for antibiotic resistance markers on the plasmid. Plasmid loss over time was expressed as per cent *Xanthomonas* cells expressing the plasmid encoded antibiotic resistance(s).

Scanning Electron and Light Microscopy

Bacterial suspensions of $\sim 10^8$ cfu/ml in sterile tap water were sprayed on young Duncan grapefruit leaves using a Crown Sprā-tool aerosol sprayer (no. 8011 power pack; Crown Industrial Products Co., Helbron, IL). Leaf discs (8 mm diameter) from infected areas were removed 7, 9, 12, 17 and 21 days after inoculation using sterile cork borers, and fixed at 4C overnight in 2% glutaraldehyde solution made in aqueous 0.066 M phosphate buffer (pH 6.8). The disks were washed and stored in the aqueous 0.066 M phosphate buffer prior to thin sectioning and microscopy. Sample processing and microscopy was as described by Brlansky *et al.* (1982).

Results

X. citri Gene Library

Based on DNA restriction profiles of plasmids from 18 arbitrarily chosen clones, the *X. citri* strain 3213 genomic DNA library appeared to contain random inserts averaging 22.5 kb in size. The genomic library consisting of 1344 clones was

maintained in *E. coli* strain DH5α. (A representative library carrying inserts averaging 22.5 kb in size requires ca. 700 clones [Sambrook et al., 1989]). The plasmids transferred at an average frequency of 5.5 X 10^{-5} per recipient into *X.c.* pv. *citrumelo* 3048.

Pathogenicity Phenotypes of pSS10.35

Five hundred independent transconjugants of *X.c.* pv. *citrumelo* 3048 containing recombinant plasmids which contained *X. citri* 3213 DNA were inoculated onto *C. paradisi* cv. Duncan grapefruit leaves at $\sim 10^8$ cfu/ml. One transconjugant out of the 500 tested (pSS10.35, 20.0 kb in size) was found to induce raised, canker-like lesions on grapefruit leaves after repeated inoculations. Plasmid pSS10.35 was again mated from *E. coli* DH5α into *X.c.* pv. *citrumelo* 3048 and retested on grapefruit leaves, with the same results. To demonstrate that pSS10.35 carried a *trans*-acting virulence gene(s), plasmid DNA was extracted from *X.c.* pv. *citrumelo* 3048 transconjugants isolated from inoculated citrus leaves, reintroduced into *E. coli* DH5α cells by transformation, and again mated into *X.c.* pv. *citrumelo* 3048. The resulting transconjugants also induced canker-like lesions when inoculated onto citrus leaves. Based on restriction enzyme digestions at the beginning and end of these manipulations, pSS10.35 appeared to be unaltered. Inoculations of citrus with low cell numbers (10^5 cfu/ml) of *X.c.* pv. *citrumelo* 3048/ pSS10.35 gave results comparable to those obtained at high cell numbers, except that low inoculation concentrations gave rise to well-separated, individual raised lesions.

Strains belonging to *X.c.* pvs. *alfalfae* and *cyamopsidis* are both compatible with citrus and cause mild water-soaked lesions on the foliage under greenhouse conditions (Gabriel et al., 1988). Introduction of pSS10.35 into strains of these pathovars followed by inoculation of grapefruit leaves resulted in corky, erumpent lesions similar to those obtained with *X.c.* pv. *citrumelo* 3048/pSS10.35. When pSS10.35 was introduced into *X. phaseoli* and *X.c.* pv. *malvacearum* strains (neither is able to grow on citrus), no visible symptoms were obtained. Even at high inoculum cell densities ($\sim 10^8$ cfu/ml), the transconjugants of these incompatible strains containing pSS10.35 were unable to incite any disease symptoms on citrus or multiply *in planta*, indicating that pSS10.35 does not carry genes capable of extending the host range of these strains to include citrus. Introduction of pSS10.35 into *X.c.* pv. *citrumelo* 3048 changed the reaction of the strain on bean (inoculated at 10^8 cfu/ml) from water-soaking to a hypersensitive response (HR), indicative of avirulence (*avr*) gene function on bean. Similarly, introduction of pSS10.35 into *X.c.* pv. *cyamopsidis* 13D5 changed the reaction of the strain on guar (inoculated at 10^8 cfu/ml) from water-soaking to little or no symptom development, again indicative of avirulence (*avr*) gene function on guar.

Surface Morphology and Histology of Lesions Caused on *C. paradisi* cv. "Duncan" Leaves.

Lesions induced by transconjugants of *X.c.* pvs. *alfalfae*, *citrumelo*, and *cyamopsidis* with and without pSS10.35 on citrus were compared with those caused by *X. citri* in both light and scanning electron microscopy studies. *X.c.* pv. *citrumelo* 3048

induced relatively flat-surfaced lesions that were easily distinguished from the pustules elicited by *X. citri* 3213 (Brlansky et al., 1982) or *X.c.* pv. *citrumelo* 3048/pSS10.35 strains. Lesions induced by *X.c.* pv. *citrumelo* 3048/pSS10.35 were indistinguishable from those induced by *X. citri* 3213 in external surface morphology (Fig. 2-1) and histology (Fig. 2-2). Host cell proliferation to the extent of rupturing the epidermal layer seemed to be of the same magnitude in both cases. Additionally, hypertrophy of the spongy mesophyll rather than hyperplasia seemed to be the main cause of pustule formation in both cases. *X. citri* 3213 and *X.c.* pv. *citrumelo* 3048/pSS10.35 induced lesions characterized by a raised epidermis which ruptures, exposing the upper palisade tissue, whereas in lesions induced by *X.c.* pv. *citrumelo* 3048, the epidermal cells seemed intact and no such rupture was observed (Fig. 2-2).

Molecular Characterization of pSS10.35 and Localization of *pth*A

A partial restriction map of pSS10.35 (20.0 kb) is shown in Fig. 2-3. The region required to elicit canker-like lesions on citrus was localized by subcloning and transposon mutagenesis to span a 3.7 kb segment (Fig. 2-3). Transposon mutagenesis of a 16.8 kb subclone, p35KX15, was carried out to insertionally inactivate gene(s) involved in conferring the pathogenicity phenotypes on citrus, bean and guar. Ninety-nine of 120 independent insertional derivatives carried Tn*5-gus*A in the 16.8 kb subcloned fragment (and not in the vector), were mated into *X.c.* pv. *citrumelo* 3048, and were screened on plants. Fourteen of these failed to induce canker-like lesions on citrus and failed to induce the avirulence phenotype on bean; the remaining 85 insertional derivatives induced

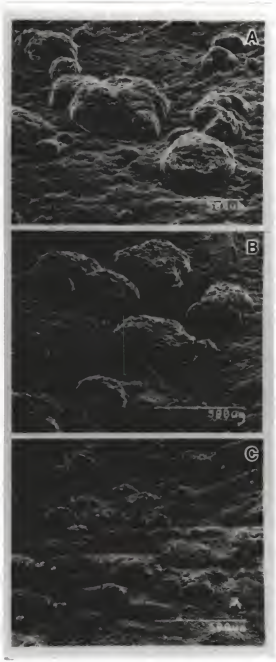


Fig. 2-1. Scanning electron micrographs of lesions induced on *Citrus paradisi* cv. "Duncan" leaves seventeen days following spray inoculations. a) *X. citri* 3213^{T}; b) *X.c.* pv. *citrumelo* 3048/pSS10.35; c) *X.c.* pv. *citrumelo* 3048.

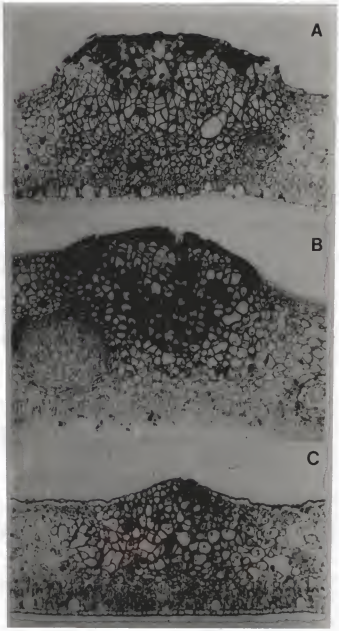
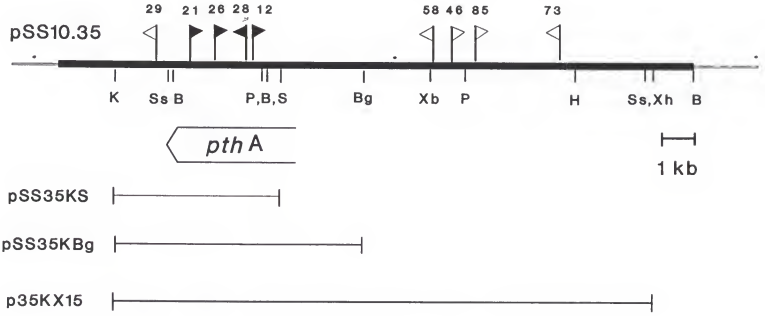


Fig. 2-2. Histopathology of *Citrus paradisi* leaves seventeen days following spray inoculations (60X magnification). a) *X. citri* 3213[T]; b) *X.c.* pv. *citrumelo* 3048/pSS10.35; c) *X.c.* pv. *citrumelo* 3048.

Fig. 2-3. Molecular characterization of pSS10.35 and of transcription of *pth*A as determined by subclones and Tn*5-gus*A insertions. Subclones pSS35KS, pSS35KBg and p35KX15 are shown below the partial restriction map of pSS10.35. Sites and orientation of Tn*5-gus*A insertions are shown above the restriction map. Arrowheads indicate the direction of the promoterless gene *gus*A1, solid arrows indicate insertions leading to loss of gene function. Numbers above the flags indicate some of the insertional derivatives characterized. (B) *Bam*HI, (Bg) *Bgl*II, (H) *Hin*dIII, (K) *Kpn*I, (P) *Pst*I, (S) *Sal*I, (Ss) *Sst*I, (Xb) *Xba*I, (Xh) *Xho*III.


pSS10.35
29
21
26
28
12
58
46
85
73
K
Ss B
P,B,S
Bg
Xb
P
H
Ss,Xh
B
pth A
1 kb
pSS35KS
pSS35KBg
p35KX15

canker-like lesions on citrus and avirulence on bean. All 14 Tn*5*-*gus*A inserts were mapped to the 3.7 kb fragment; four of these are localized in Fig. 2-3. When these 14 p35KX15::Tn*5*-*gus*A insertional derivatives were introduced into *X.c.* pv. *cyamopsidis* strains, none of the insertional derivatives induced canker-like lesions on citrus, or reduced virulence on guar. This region has at least one gene, designated *pth*A (pathogenicity A) that appeared to operate *in trans* for virulence enhancement of strains compatible with citrus and for avirulence of the same strains on other homologous hosts. No subclone, nor any of the 99 insertional derivatives tested, separated the two phenotypes (virulence enhancement on citrus and avirulence on bean and guar).

Marker Exchange Mutagenesis

In order to determine the role of the *pth*A locus in *X. citri* pathogenicity on citrus, marker-exchange mutagenesis was carried out. Transfer of displacement vector pUFR049 into *X. citri* 3213/p35KX15::Tn*5*-*gus*A aided the recovery of marker-exchanged mutants. The frequency of marker-exchange varied with clones, position of transposon insertion and strains, but was generally in the range of 1-10% of the colonies recovered in the procedure. A Southern blot analysis and pathogenicity test of one representative marker-exchanged mutant, *X. citri* B21.2 (*pth*A::Tn*5*-*gus*A), are shown in Figs. 2-4 and 2-5, respectively. In Figure 2-4, the band corresponding to the 3.5 kb *Bam*HI fragment of wild type *pth*A is indicated by an arrow in both lanes 1 (pSS35KS) and 2 (*X. citri* 3213 total DNA). The heavier intensity of the band in lane 1 is due to overloading the DNA sample in lane 1 relative to the amount of the identical band loaded

Fig. 2-4. Southern hybridization showing marker-exchange mutagenesis of *pth*A in *X. citri*. Lane 1: *X.citri* B21.2 (*pth*A::Tn*5*-*gus*A mutant derivative of 3213). Lane 2: *Bam*HI digested genomic DNA of *X.citri* 3213^{r}. Lane 3: *Bam*HI digested pSS35KS DNA. Southern blot probed with 3.7 kb *Sst*I-*Sal*I fragment of pSS35KS carrying *pth*A. L: ^{32}P-labelled molecular weight markers (1 kb multimers from 3 to 12kb shown). V = vector plus pSS35KS junction fragments to the *Bam*HI sites. Arrows indicate Tn*5*-*gus*A affected fragments.

in lane 2. The multiple hybridizing bands in lanes 2 and 3 indicate the presence of at least four similarly sized, and one larger sized, *Bam*HI DNA fragments homologous with the 3.7 kb probe. Note the disappearance of the 3.5 kb *pth*A *Bam*HI fragment in lane 3 and the appearance of a new 12.9 kb band (Tn*5*-*gus*A is 9.4 kb in size, and has no *Bam*HI sites). The blot shown in Fig. 2-4 was rehybridized with radiolabelled Tn*5*-*gus*A as a probe, and revealed homology only to the 12.9 kb band (rehybridized blot not shown), demonstrating insertion of Tn*5*-*gus*A by marker exchange in the appropriate 3.5 kb (*pth*A) *Bam*HI fragment.

Marker exchange of *pth*A::Tn*5*-*gus*A in *X. citri* B21.2 resulted in a complete loss of pathogenic symptoms on cv. "Duncan" grapefruit, even at 10^8 cfu/ml inoculation levels (Fig. 2-5). Furthermore, mutant B21.2 lost the ability to induce a heterologous hypersensitive response (HR) on non-host plants of *Phaseolus vulgaris* cv. "California Light Red" (data not shown). Complementation analysis of the *pth*A::Tn*5*-*gus*A mutation was carried out by introducing pSS35KBg (7.7 kb insert) into *X. citri* B21.2. Restoration of the phenotype to the wild type response was observed on inoculated cv. Duncan grapefruit leaves with B21.2/pSS35KBg at both high and low inoculum levels (Fig. 2-5). Restoration of the heterologous HR response was also observed.

The direction of transcription of *pth*A indicated in Figure 2-3 was determined by β-glucuronidase (GUS) assays of marker exchanged mutants grown *in planta* and in PYGM broth. Expression of *pth*A was observed in B21.2 grown *in planta* and in broth. The 5' end of *pth*A lies between the *Sal*I and *Bgl*II restriction sites, and the 3' end lies between the *Sst*I and *Kpn*I sites, based on partial DNA sequence and subcloning analyses.

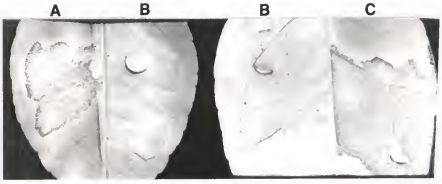


Fig. 2-5. Lesions induced on *Citrus paradisi* leaves one week following infiltration with 10^8 cfu/ml of *X.citri* 3213, marker-exchanged mutant B21.2 and complemented mutant. a) *X.citri* 3213^r; b) B21.2; and c) B21.2/pSS35KBg.

Transconjugants of *X.c.* pv. *citrumelo* 3048 or *X.c.* pv. *cyamopsidis* 13D5 strains containing all the subclones of pSS10.35 ending at either the *Sal*I site or the *Sst*I site (adjoining the *Bam*HI sites) failed to elicit canker-like lesions when inoculated onto citrus. This indicated that the *pth*A region spanned (and was not delimited by) the *Sal*I and *Sst*I sites adjoining the *Bam*HI site.

Growth Kinetics *in planta*

Growth kinetics of *X. citri* 3213, *X. citri* B21.2 (*pth*A::Tn*5*-*gus*A) and *X. citri* B21.2/pSS35KBg were studied in cv. "Duncan" grapefruit leaves. Results are presented in Fig. 2-6. Growth of marker-exchange mutant strain B21.2 was reduced by two orders of magnitude after 15 days *in planta* as compared to the wild type strain 3213. Tn*5*-*gus*A insertion in *pth*A also led to a rapid decline in the bacterial population as compared to the wild type, which survived for a longer time in leaf tissue. Unexpectedly, growth kinetics of the complemented strain B21.2/pSS35KBg was found to be the same as that of B21.2 (Fig. 2-6), even though well-separated, raised, canker-like lesions were observed. Percent retention of pSS35KBg over time was followed during the course of the *in planta* growth kinetics experiment. In several repetitions of the experiment, more than 80% of cells extracted from citrus leaf tissue had lost pSS35KBg within 24 hours of inoculation, based on loss of the plasmid antibiotic markers.

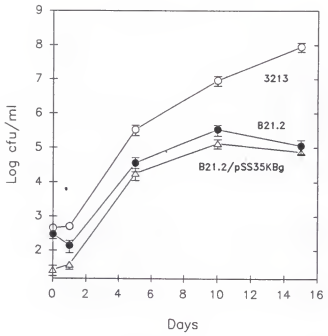


Fig. 2-6. Growth kinetics *in planta* of wild-type *X.citri* 3213^{T}, marker exchange mutant B21.2 and mutant B21.2 complemented by cloned wild-type DNA on pSS35KBg.

Discussion

Screening of a recombinant gene library of the highly virulent Asiatic canker pathogen *X. citri* 3213 in the mild pathogen *X.c.* pv. *citrumelo* 3048 on citrus resulted in the isolation of a clone, pSS10.35, that conferred the ability to elicit canker-like symptoms on citrus. This virulence enhancement on citrus was conferred to strains of two other pathovars tested (*X.c.* pvs. *alfalfae* and *cyamopsidis*) that are compatible with citrus, but was not conferred to strains of two other xanthomonads that are incompatible with citrus (*X.c.* pv. *malvacearum* and *X. phaseoli*). There was no evidence that pSS10.35 extended the host range of the incompatible strains to citrus. In contrast with attempts to extend host range (Sawczyc et al., 1989), the virulence enhancement approach requires that both the DNA donor and recipients are compatible with the same host. Nevertheless, the enhanced virulence of pSS10.35 was host-specific. When present in *X.c.* pvs. *alfalfae, citrumelo* or *cyamopsidis,* pSS10.35 conferred enhanced virulence only to citrus, and avirulence in interactions with other homologous hosts.

Transposon mutagenesis and subcloning allowed localization of the pathogenicity /avirulence activities to a 3.7 kb fragment. All fourteen Tn*5-gus*A inserts in this region abolished both virulence and avirulence phenotypes; all 85 Tn*5-gus* inserts outside of the region left both phenotypes intact. A marker exchange mutant of *X. citri* 3213^{r}, B21.2, did not elicit any canker-like symptoms on citrus, even when inoculated at relatively high cell densities. We conclude that pSS10.35 carries at least one gene, *pth*A, that is necessary, but not sufficient, for citrus canker disease. We found no evidence for a second virulence (*hrp*) or avirulence (*avr*) locus on the 3.7 kb fragment, although

complete DNA sequencing will be required to prove that the virulence enhancement and avirulence properties are pleiotropic effects of the Asiatic citrus canker pathogenicity gene, *pth*A.

The marker exchange mutant *X. citri* B21.2 was greatly affected in both *in planta* growth and ability to induce a pathogenic reaction on citrus, as compared to the wild type strain, *X. citri* 3213. Complementation of the disease inducing ability, but not the reduced growth *in planta* of B21.2 was achieved with pSS35KBg, carrying the *pth*A locus. We were surprised that pSS35KBg did not even slightly restore growth in citrus (Fig. 2-6), despite the clear restoration of pathogenicity symptoms in B21.2 at high inoculation levels (Fig. 2-5). Even at low inoculation levels, B21.2/pSS35KBg elicited well-separated, raised, canker-like lesions on citrus. The reduction of *in planta* growth of B21.2 caused by the Tn*5-gus*A insertion may be a secondary (perhaps polar) effect of the transposon. Alternatively, the reduction in growth may be a direct effect on *pth*A and growth *in planta* might be restored if pSS35KBg were stabilized. Although the vector pUFR042 is more than 95% stable *in planta*, pSS35KBg was lost rapidly *in planta*. The cause of the instability is not known.

Genes required for virulence, but not involved in the heterologous hypersensitive response (HR) (Klement, 1963) have been cloned from *X.c.* pv. *campestris* (Daniels et al., 1984, Turner et al., 1985), *Pseudomonas syringae* pv. *tomato* (Cuppels, 1986), *P.s.* pv. *pisi* (Malik et al., 1987), *P. solanacearum* (Boucher et al., 1987), *P. syringae* pv. *syringae* (Willis et al., 1990), and others. Genes affecting both pathogenicity and the heterologous HR phenotypes (ie., *hrp* genes [Lindgren et al., 1986]) have been cloned

from a wide variety of gram-negative bacteria, including *Pseudumonas solanacearum* (Boucher et al., 1987, Huang et al., 1990), *P. syringae* pv. *syringae* (Niepold et al., 1985), *P. s.* pv. *phaseolicola* (Lindgren et al., 1986), *P.s.* pv. *tomato* (Cuppels, 1986), *P.s.* pv. *pisi* (Malik et al., 1987), *X.c.* pv. *campestris* (Kamoun et al., 1990), *X.c* pv. *vesicatoria* (Bonas et al., 1991), *X.c.* pv. *citrumelo* (M. Kingsley and D. Gabriel, unpublished), *Erwinia amylovora* (Bauer and Beer, 1987). Based on hybridization and cross-complementation analyses, such genes appear to be highly conserved, even at the family level (Boucher et al., 1987, Lindgren et al., 1988). The 3.7 kb *pth*A locus is not conserved at the genus level. Although *X. citri* 3213 carries at least four other DNA fragments homologous with the 3.5 kb *Bam*HI fragment of pSSKBg (Fig. 2-4), *X.c.* pvs. *citrumelo* 3048, *alfalfae* KX-1 and *cyamopsidis* 13D5 carried no homologous bands (based on unpublished results of hybridizations similar to those shown in Fig. 2-4). In these strains, which lack the *pth*A locus, *pth*A appeared to act as an add-on pathogenicity/avirulence factor. The formation of cankers and rupturing of epidermal layers leading to oozing out of bacteria on the leaf surface (Lawson et al.,1989) might be an important aid in the dispersal of *X. citri* strains. Strains of *X.c.* pv. *citrumelo* rarely elicit lesions on citrus leading to rupture of leaf epidermis. As a consequence, they should not be able to emerge on the leaf surface in cell numbers comparable with those of *X. citri* strains. It is known that in field situations, *X.c.* pv. *citrumelo* strains disperse very poorly on citrus compared with *X. citri* (Gottwald and Graham, 1990). Strains belonging to *X. citri* are narrow-host range pathogens but well adapted to many varieties of citrus. They occur worldwide wherever citrus is grown (Schoulties et al.,

1987) and are highly clonal in their population structure (Gabriel et al., 1988, 1989; Graham et al., 1990). The genetic similarity of these pathogens implies the presence of virulence genes of high selective value on citrus. (For general discussions of the role of virulence factors on the population structures of microbial plant and animal pathogens, refer Gabriel, 1989 and Selander, 1985, respectively). Although we have not ruled out the possibility of more than one virulence gene on pSS10.35, at least one of them, *pth*A, may be partly responsible for the observed clonality of *X. citri* world-wide.

Virulence often has been argued to be a feature of a pathogen over and above its basic compatibility with a host (Kennedy and Lacy, 1982, Gabriel, 1989). Pathogens with a broad host range might become preferentially and better adapted to one host plant by slow evolutionary selection or by horizontal gene transfer. Little is known about the actual frequencies of horizontal gene transfer among bacteria in natural environments (for reviews, see Stotzy and Babich, and Trevors et al., 1987), except that it occurs (Schofield et al., 1987). Since *X. citri* and *X.c.* pv. *citrumelo* strains can occupy the same niche (e.g., citrus), there is an obvious potential for exchange of genetic material. Since the *pth*A locus is confined to a relatively small, 3.7 kb fragment, it could be readily transferred. If *pth*A conferred a selective advantage to *X.c.* pv. *citrumelo*, its eventual transfer from *X. citri* might be predictable. Some factors affecting its transfer potential might be 1) the potential frequency of transfer of the *pth* locus (by transformation, transduction or conjugation); 2) frequency of simultaneous colonization of citrus by both species; and 3) reproductive advantage conferred by the locus on the

recipient strain under normal field conditions. All these factors are experimentally tractable, and could lend insight into the origins of new pathogen epidemics.

The most fit combinations of host-specific virulence genes available in the genus *Xanthomonas* may not be present in any one strain at a given time. This may be particularly true if the host is a recently developed hybrid, such as the rootstock Swingle Citrumelo, a newly released hybrid of two plant genera (*Citrus paradisi* X *Poncirus trifoliata*). Strains of *X. c.* pv. *citrumelo* seem to be broad host range pathogens that are not well adapted to citrus and are not known to be highly virulent or well adapted to any plant species; even on Swingle Citrumelo they spread only on juvenile tissue (Gottwald and Graham, 1990). They have never been observed in other citrus-growing regions of the world. These strains may themselves be natural recombinants selected by the new citrus variety. Had there not been an aggressive program for citrus canker eradication in Florida (Schoulties et al., 1987), it is possible that the strains of *X. citri* and *X.c.* pv. *citrumelo* found in Florida could have been simultaneously present on the same citrus hosts, and allow for an even more fit combination of virulence genes specific for citrus. It would be interesting to learn if *X.c.* pv. *citrumelo* carrying *hsv*A is more virulent on citrus than *X. citri* 3213 in field situations. However, it seems unlikely that field studies can be undertaken in this case, since the field release of such a strain would not likely gain the required state or federal regulatory agency approvals.

CHAPTER III
A *XANTHOMONAS CITRI* PATHOGENICITY GENE, *pth*A, PLEIOTROPICALLY ENCODES GRATUITOUS AVIRULENCE ON NON HOSTS

Introduction

The function and extent of the roles avirulence (*avr*) genes play in plant-associated microbes have been a subject of speculation (Gabriel and Rolfe, 1990; Keen, 1990). In terms of biological function, *avr* genes determine race specificity by limiting the range of cultivars, and occasionally species and genera, a pathogen may attack. Race- and cultivar-specific interactions can usually be shown to require the presence of specific resistance (*R*) genes in the host and these negative (incompatible) interactions are termed gene-for-gene interactions. Gene-for-gene interactions are superimposed on a basic ability to parasitize (Ellingboe, 1976) and are generally associated with a plant defense response.

Plant defense responses are also observed when pathogens are inoculated onto non-hosts. Single cloned avirulence genes isolated from a pathogen of one host-species can cause an otherwise virulent pathogen of another host-species to become avirulent on its own host (Kobayashi et al., 1988; Whalen et al., 1988). These and other observations have led to the suggestion that avirulence genes may also determine host range above the race level (for example, refer Keen, 1990; Keen and Staskawicz, 1988). Alternatively,

it has been argued that the role of avirulence genes in non-host incompatibility is generally gratuitous and that positive functioning genes play the major role in determining host-species specificity (Gabriel, 1989). According to this argument, positive acting host-range genes, such as the host-specific nodulation (*hsn*) genes of *Rhizobium* spp. (Djordjevic et al., 1987; Martinez et al., 1990), and the host-specific virulence genes (*hsv*) genes of *Xanthomonas* spp. (Waney et al., 1991) and *Pseudomonas* spp. (Ma et al., 1988; Salch and Shaw, 1988) may play the predominant roles in determining host range.

Inactivation of known avirulence genes has not been reported to result in the loss of the non-host HR, possibly because of the virtually limitless numbers of non-hosts that could be tested. Also, a large number of *avr* genes may be involved in many non-host HR reactions and elimination of any one *avr* gene would not affectthe epistatic effects of other *avr* genes. In one case which likely involved an *avr* gene, both chemically- and transposon-induced mutants of *Erwinia rubrifaciens* were obtained that had lost the ability to induce the heterologous HR on tobacco, and yet were as pathogenic to walnut (the normal host) as the wild type (Azad and Kado, 1984). The transposon mutants likely affected a single *avr* locus, indicating that some heterologous HR reactions are due to single *avr* gene to *R* gene interactions. Although there was no obvious pathogenicity of the resulting *E. rubrifaciens* mutants on tobacco, it was not determined whether asymptomatic growth occured in tobacco.

Cloning of pathogenicity locus, *pth*A, essential for canker-inducing ability of *X. citri* on citrus was described in chapter 2. In this chapter, I report further

characterization of *pth*A and show that it (i) pleiotropically functions in *X. citri* to elicit the non-host hypersensitive response (HR) of bean cv. Calif. Lt. Red, (ii) functions as an avirulence gene in *X. phaseoli* and *X.c.* pv. *malvacearum*, (iii) is physically similar to other avirulence genes of *Xanthomonas* spp.; and (iv) when inactivated in *X. citri*, the non-host HR is eliminated on bean, but growth *in planta* is not substantially affected, and host-range is not thereby extended.

Materials and Methods

Bacterial Strains, Culture Media and Plasmids

Sources and characteristics of all strains of *Xanthomonas* spp. used in this study were described in chapter 2. *Escherichia coli* DH5α (Bethesda Research Laboratories; BRL) was the cloning host for all plasmids used in this work. All *Xanthomonas* spp. strains were cultured on PYGM medium at 30°C (Gabriel et al., 1989) and all *E. coli* strains were cultured on LB medium at 37°C (Miller, 1972). Antibiotics were used at concentrations as described in the previous chapter.

The small, stable shuttle vector pUFR027 (9.4 kb, Nm^R) (DeFeyter et al., 1990) was used to clone pSS10.35. The subclones pSS35KBg (described in the previous chapter), pSS35KS1, and pSS35BP3 contained variously sized fragments of pSS10.35 in pUFR042 (8.5 kb, Gm^R, Nm^R) (DeFeyter and Gabriel, 1991).

Bacteriological and Recombinant DNA Techniques

Triparental matings were used to transfer clones from *E. coli* DH5α to various Sp^R *Xanthomonas* spp. strains using pRK2013 (Ditta et al., 1980) or pRK2073 (Leong et al., 1982) as helper strains. Standard recombinant DNA techniques (Sambrook et al., 1989) were followed for various cloning and restriction mapping experiments. Southern blot analyses were performed using nylon membranes according to the manufacturer's recommendations. Probe DNA was radiolabelled with ^{32}P-dCTP using random primers (Feinberg and Vogelstein, 1983). The membranes were treated, hybridized and washed as described (Lazo et al., 1987).

*Bal*31 Deletion Subcloning

Digestion of DNA from pSS35KBg was carried to completion with K*pn*I and H*ind*III to excise the insert (7.7 kb) from the vector pUFR042. B*al*31 nuclease was titrated to remove ~130 bp/end/min and used to construct deletions varying in size as described (Sambrook et al., 1989). A time point which yielded 3.5-5.0 kb fragments was chosen and the DNA was treated with S1 nuclease as described (Sambrook et al., 1989). Subsequently, the termini of the fragments were modified to blunt them using Klenow fragment and the inserts ligated to S*ma*I linearized pUFR047 DNA (an unpublished 8.6 kb, Gm^RAp^R derivative of pUFR042). The deletion subclones obtained upon transformation of *E. coli* DH5α cells were analyzed by restriction mapping and conjugally transferred to various *Xanthomonas* spp. strains. The resulting

transconjugants were screened on *Citrus paradisi* cv. 'Duncan' (Grapefruit), *Phaseolus vulgaris* cv. 'California Light Red', *Cyamopsidis tetragonoloba* USDA PI 215590, and *Gossypium hirsutum* cv. 'Acala 44' congenic resistant lines (DeFeyter and Gabriel, 1991) for *pth*A activity. The inserts from two deletion clones with *pth*A activity, pZit34 and pZit45, were subcloned into pUC119 (Vieira and Messing, 1987) to yield pZit34.119 and pZit45.119, respectively. These subclones were used for DNA sequencing and as sources of probe DNA for Southern hybridizations.

Plant Inoculations and *In Planta* Bacterial Growth Curves

Bean, guar and cotton plants were grown under greenhouse conditions and then transferred to growth chambers three days prior to inoculation and maintained under conditions as described in previous chapter. All citrus plants were grown and inoculated in quarantine greenhouse facilities at the Division of Plant Industry, Florida Department of Agriculture, Gainesville, Florida. *In planta* bacterial growth kinetics experiments and pathogenicity assays involving *X. citri* 3213^{T} (ATCC 49118) (Gabriel et al., 1989), B21.2, and their transconjugants were all performed in the quarantine greenhouse facilities. Inoculations with bacterial suspensions in sterile tap water standardized to 10^{8} cfu/ml were performed by pressure infiltration as described previously, and inoculations of cotton as described by Gabriel et al. (1986). At least three replications of each inoculation of a single strain on 2-3 plants were included in a single experiment. Each experiment was repeated at least three times. Data shown are from a single

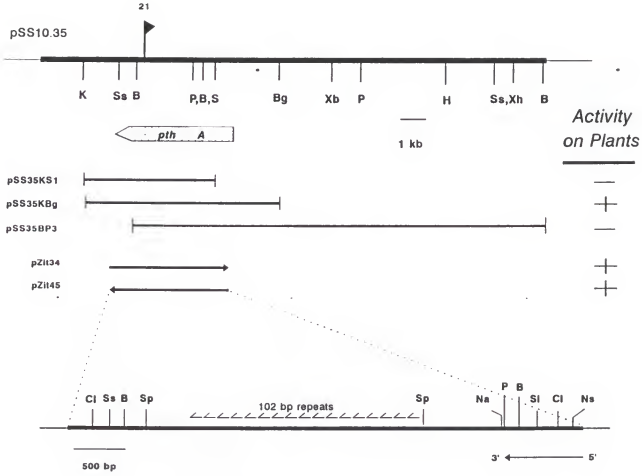
representative experiment. To monitor the growth of *Xanthomonas* spp. strains in bean cv. Calif. Lt. Red, bacterial suspensions were adjusted to 10^6 cfu/ml in sterile tap water and pressure-infiltrated into fully expanded abaxial leaf surfaces. Leaf sections of 1 cm^2 were taken with sterile cork-borers from non-injured areas of the leaves, macerated in 1 ml sterile tap water and various dilutions plated on appropriate selective antibiotic containing media. Viable counts were made after 3 days of incubations and these were expressed as $\log_{10}$cfu/cm^2 of inoculated leaf surface.

Results

Localization and Subcloning of *pth*A From pSS10.35

Gene *pth*A was shown in the previous chapter to be present on a 7.7 kb DNA fragment (pSS35KBg), and the entire 3.5 kb *Bam*HI fragment (Fig. 3-1) was found to be essential for *pth*A activity. A combination of exonucleases were used to generate deletion subclones of pSS35KBg in order to further delimit the functional boundaries of *pth*A. Two plasmids carrying inserts in opposite orientations, pZit34 and pZit45 (Fig. 3-1), contained the smallest inserts (4.5 kb each) that still expressed full *pth*A activity on all hosts studied. When introduced into *X.c.* pvs. *alfalfae* KX-1, *citrumelo* 3048 and *cyamopsidis* 13D5, both pZit34 and pZit45 conferred the ability to cause canker-like lesions on citrus identical to the ones conferred by pSS35KBg. While constructing the progressive deletions, twenty clones were assayed in appropriate strains for pathogenicity

Fig. 3-1. Structure and localization of *pth*A. Restriction map of pSS10.35; selected subclones and their activity on plants. pSS35KS1 is a *Kpn*I-*Sal*I fragment; pSS35BP3 is a *Bam*HI partial fragment; and the pZit clones are *Bal*31 exonuclease derivatives of pSS10.35. Flag with closed triangle shows the site of the Tn*5*-Gus insertion used to create B21.2. Subclones of pSS10.35 retaining activity on plants are shown as "+" and those not retaining the activity as "-". Detailed restriction map of pZit45 shows the number and direction of 102 bp multiple DNA repeats. Direction of transcription of *pth*A is shown by the arrows. Restriction sites are abbreviated as follows: B, *Bam*HI; Bg, *Bgl*II; Cl, *Cla*I; H, *Hin*dIII; K, *Kpn*I; Na, *Nae*I; Ns, *Nsi*I; P, *Pst*I; S, *Sal*I; Sp, *Sph*I; Ss, *Sst*I; Xh, *Xho*I.


21
pSS10.35
K
Ss B
P,B,S
Bg
Xb
P
H
Ss,Xh
B
pth A
1 kb
Activity on Plants
pSS35KS1
pSS35KBg
pSS35BP3
pZit34
pZit45
Cl Ss B
Sp
102 bp repeats
Sp
P B
Na
Sl
Cl
Ns
500 bp
3'
5'

activity on citrus and avirulence activity on various non-hosts. All of the clones had either both pathogenicity and avirulence functions intact or were non-functional for both pathogenicity on citrus and avirulence on non-citrus hosts.

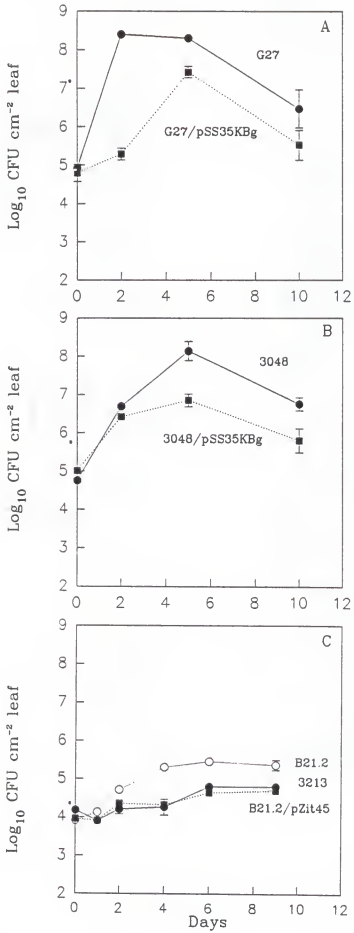
Interaction of *Xanthomonas* Strains Compatible with Bean and Guar and their Transconjugants Containing Cloned *pth*A

X.c. pv. *citrumelo* 3048/pZit45 and *X. phaseoli* G27/pZit45, when inoculated at 10^8 cfu/ml, elicited a distinct hypersensitive response (HR) on bean cv. California Light Red leaves 48 hours after inoculation. (The same strains without pZit45 are compatible on cv. Calif. Lt. Red with obvious water-soaking). In order to study the effect of *pth*A on the ability of these compatible strains to grow *in planta*, the bacterial population growth over time was monitored. As expected, a significant decrease in growth was seen in cases of *X.c.* pv. *citrumelo* 3048 and *X. phaseoli* G27 transconjugants containing cloned *pth*A (in pSS35KBg), as compared to the same strains without *pth*A (Figs. 3-2a and 3-2b). Within five days of inoculation, transconjugants of both strains were at approximately 10-fold lower concentrations *in planta* than their respective wild type strains.

Response of Bean cv. Calif. Lt. Red and Cotton cv. Ac44 Congenic Resistant Lines to *X. citri*

A non-host hypersensitive response (HR) was induced in bean cv. Calif. Lt. Red within 48 hours of inoculation with 10^8 cfu/ml of *X. citri* 3213. This response was not elicited on inoculation with 10^8 cfu/ml of the mutant B21.2 (*X. citri pth*A::Tn*5*-Gus)

Fig. 3-2. Growth of *Xanthomonas* strains in bean cv. California Light Red over time. **A.** *X. phaseoli* G27 and G27/pSS35KBg containing *pth*A; **B.** *X.c.* pv. *citrumelo* 3048 and 3048/pSS35KBg containing *pth*A; **C.** *X. citri* 3213, B21.2 *pth*A$^-$, and B21.2/pZit45 containing *pth*A. Vertical bars show the standard error at each sampling time.


A
G27
G27/pSS35KBg
B
3048
3048/pSS35KBg
C
B21.2
3213
B21.2/pZit45
Log10 CFU cm-2 leaf
Days

strain. Although B21.2 did not elicit the non-host HR, it remained non-pathogenic on bean as evidenced by a lack of growth *in planta* (Fig. 3-2c) and the lack of any visible disease symptoms 14 days after infiltrating leaves with 10^8 cfu/ml of inoculum (not shown). Cel counts *in planta* in bean cv. Calif. Lt. Red of *X. citri* strains 3213 and B21.2/pZit45 remained at almost the same levels (i.e., 10^4 to 10^5 cfu/cm^2 leaf) as inoculated. The strain B21.2 grew 1 $\log_{10}$cfu/cm^2 leaf higher than both the wild type strain 3213 and B21.2/pZit45.

Interaction of Congenic Cotton Resistant Lines With *X.c.* pv. *malvacearum* Containing Cloned *pth*A and *X. citri* (*pth*A::Tn5-Gus)

A set of nine different resistant lines of cotton, congenic with cv. Acala 44 by backcrossing, were inoculated with 10^8 cfu/ml inocula of *X.c.* pv. *malvacearum* strains H and N and *X.c.* pv. *malvacearum* N/ pZit45 (Table 3-1). *X.c.* pv. *malvacearum* H and transconjugants of *X.c.* pv. *malvacearum* N containing cloned avirulence genes from *X.c.* pv. *malvacearum* H (DeFeyter and Gabriel 1991) served as controls for these sets of experiments. Transconjugants of *X.c.* pv. *malvacearum* N containing cloned *pth*A (in pZit45) were incompatible on some but not all resistant lines (Table 3-1). *X.c.* pv. *malvacearum* N/pZit45 elicited a weak HR on Ac44 lines AcB5-82. The water soaking symptoms on Ac44 line B4 were markedly reduced indicating weak compatibility with *X.c.* pv. *malvacearum* N/pZit45. By contrast, disease symptoms on line BIn1 were totally abolished indicating an incompatible, but non-HR response. The other six resistant lines (*viz.*, lines AcBl, AcB2, AcB5, Acb6, Acb7 and AcBIn3) developed either

Table 3-1. Specificity of *pth*A on cotton cv. Acala 44 and nine congenic resistant lines.

Xanthomonas strains	Cotton cv. Acala44 congenic resistant lines									
	Ac44	B1	B2	b7	B5	b6	b7	B5-82	BIn1	BIn3
X.c. malvacearum N	+	+	+	+	+	+	+	+	+	+
X.c. malvacearum N/pZit45	●	-	-	+*	-	●	●	-*	O	-

\+ means a compatible interaction, as indicated by a watersoaking lesion

\- means an incompatible interaction, as indicated by a hypersensitive response (HR)

O means a null response, as indicated by lack of any visible plant reaction

rapid and distinct HR or water soaking lesions. However, the HR appeared slower (24 hours later) on line AcB5 compared to those on lines AcBIn3, AcB1, AcB2 and AcB5-82. The water soaking symptoms of *X.c.* pv. *malvacearum* N/pZit45 of lines Acb6 and Acb7 were of the same order as those seen in Acala 44, which is devoid of any known resistance genes against *X.c.* pv. *malvacearum*. In inoculations of these lines with 10^8 cfy/ml of *X. citri* B21.2, an HR was observed on all the lines including the line Acala 44 (Table 3-1).

Gene *pth*A is Homologous to a Number of Different *avr* Genes From *Xanthomonas*

Based on the fact that *pth*A conferred avirulence to *Xanthomonas* strains on hosts other than citrus, I investigated whether this gene was similar to any previously cloned avirulence gene(s) from plant pathogenic bacteria. Gene *pth*A (Fig. 3-1) was of similar size and restriction profile (from the *Bam*HI site at its 5'-end to the *Sst*I site at its 3'-end) to *avr*Bs3 of *X.c.* pv. *vesicatoria* (Bonas et al., 1989) (refer Fig. 3-1). Similarity of size and of restriction sites was also found with *avr*B4, *avr*b6, *avr*b7, *avr*BIn3, *avr*B101 and *avr*B102 cloned from *X.c.* pv. *malvacearum* H (DeFeyter and Gabriel, unpublished). Southern hybridization revealed that *pth*A hybridized to all these *avr* genes (Fig. 3-3, some data not shown). As with *avr*Bs3, the internal region of *pth*A also contained direct multiple repeats. This was evidenced by the presence of a consistent sized ladder of ca. 100 bp (not shown) upon partial digestion of pZit45.119 DNA with *Bal*I. DNA sequencing of the repeats (data not shown) revealed that, as in the case of *avr*Bs3, each repeat was 102 bp in size and that the repeats were nearly identical in their sequences

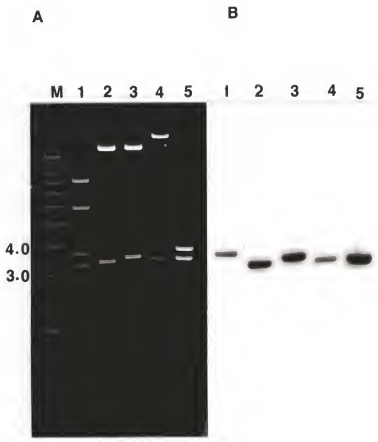


Fig. 3-3. Southern hybridization of *Xanthomonas avr* genes with *pth*A. Lanes: M, molecular weight marker (1 kb ladder); Lanes 1-3, *Bam*HI digested DNA of pRdF136.8, pRdF139.4, and pRdF139.13 containing *avr*BIn, *avr*B101, and *avr*b7 respectively from *X.c.* pv. *malvacearum*; Lane 4, *Bam*HI digested DNA of pL3XV1-6 containing *avr*Bs3 from *X.c.* pv. *vesicatoria*, and Lane 5, *Bam*HI digested DNA of pZit45 containing *pth*A from *X. citri*. **A.** Ethidium Bromide stained gel. **B.** Autoradiograph of a Southern blot of cloned *avr* genes (shown in Fig. 3-3a) hybridized to *pth*A probe DNA in pZit45.119.

and highly similar to the ones found in *avr*Bs3 from *X.c.* pv. *vesicatoria* (Bonas et al., 1989), and in *avr*B4 and *avr*b6 of *X.c.* pv. *malvacearum* (Gabriel, unpublished).

Discussion

Pathogenicity locus *pth*A is essential for the elicitation of Asiatic citrus canker symptoms by *Xanthomonas citri* 3213^{T} as described in the previous chapter. A marker-exchange mutation of *pth*A (carrying Tn5-Gus) in *X. citri* 3213 totally abolished the pathogenicity of the resulting strain (B21.2) on citrus and affected growth *in planta*. Strain B21.2 also failed to elicit an HR on bean, a non-host, but did elicit a normal HR on another non-host, cotton. Judging from the host reaction on bean and citrus, gene *pth*A could be considered as a *hrp* (Lindgren et al., 1986) gene. It has previously been pointed out that reliance on a single non-host plant species for heterologous HR tests may lead to erroneous conclusions about virulence (Azad and Kado, 1984). Observations in this study confirm this and point out the need for inoculating a number of non-host plants in mutational analyses of genes affecting virulence. Gene *pth*A was clearly distinguished from a *hrp* gene which, on mutation, would lose the ability to induce HR on all non-hosts.

Screening of congenic cotton resistant lines with transconjugants of the virulent cotton pathogen *X.c.* pv. *malvacearum* N containing *pth*A showed that *pth*A conferred cultivar specific avirulence (Table 3-1). The specificity conferred by *pth*A was different from that of all previously cloned *avr* genes from *X.c.* pv. *malvacearum* strain H

(Gabriel et al., 1986; DeFeyter and Gabriel, 1991). Results from the present study strongly suggested a gene-for-gene avirulence function of *pth*A. In *X. citri*, *pth*A played a role mainly in the elicitation of host symptoms which is entirely consistent with its role as a functional *avr* gene.

Physical characterization of *pth*A revealed several lines of evidence that *pth*A was highly similar to at least seven other *avr* genes of *Xanthomonas* spp. As with *avr*Bs3, 102 bp multiple direct repeats of DNA form the central core region of the gene. The presence of direct repeats resulted in an unusually large size of 4.5 kb for a prokaryotic gene. The fact that none of the 14 transposon insertional derivatives of *pth*A or the 20 B*al*31 deletion subclones used in cloning of *pth*A were able to separate the pathogenicity function on citrus from the avirulence function on non-citrus hosts suggested that the same open reading frame was responsible for both the functions.

According to the genetic selection theories (Falconer, 1986), it is expected that in the absence of a selection pressure, avirulence genes may eventually be lost from the pathogen population (Day, 1974; Van der Plank, 1968). However, certain avirulence genes in pathogen populations increase in frequency in the absence of the corresponding resistant genes (Van der Plank, 1975; Watson, 1980). Such phenomena have been explained as "stabilizing" selection, based on a hypothetical pleiotropic function of *avr* genes (Van der Plank 1963, 1968; Crill, 1977, Leonard and Czochor, 1980; Parlevilet, 1981). Genetic selection theories (Grant and Archer, 1983) and genetic segregation analyses of fitness vs. avirulence function (Bronson and Ellingboe, 1986) have been used to test these ideas. The lack of strong evidence for "stabilizing" selection and the high

mutation frequencies of *avr* genes led to the suggestion that most avirulence genes are gratuitous (Gabriel, 1989). Only one *avr* gene, from *X.c.* pv. *vesicatoria* (*avr*Bs2) has been reported to contribute to the fitness of a pathogen on its compatible host (Kearney and Staskawicz, 1990). Similar investigations in several laboratories with other *avr* genes have failed to reveal results similar to those found with *avr*Bs2. Gene *pth*A may contribute to the fitness of *X. citri* by inducing the citrus epidermis to rupture, thereby aiding dispersal. Such non-gratuitous pleiotropic functions, demonstrated or suggested, are exceptional.

Unlike gene *pth*A, most avirulence gene from plant pathogens have been identified based on interactions with plants carrying specific resistance genes. No races of *X. citri* are known and no genes governing resistance in citrus have been documented. *X. citri* has a very wide host range, covering all citrus varieties (Stall and Seymour, 1983). Although *pth*A of *X. citri* can exhibit cultivar-specific avirulence (in *X.c* pv. *malvacearum* on cotton congenic resistant lines), it is not known to function for avirulence (cultivar-specific or otherwise) within Rutaceae. Gene *pth*A caused a heterologous HR on bean plants when introduced into *Xanthomonas* strains compatible on this host. Gene *pth*A might, therefore, be thought to limit host range on bean. Marker exchange of *pth*A in *X. citri* eliminates the heterologous HR on bean but there is not a substantial increase in growth or any symptoms of pathogenicity on bean. One could argue that other, non-HR inducing *avr* genes are present, which limit host range of *X. citri* on bean. For example, in the present study *pth*A in *X.c.* pv. *malvacearum* resulted in a non-HR, host-gene-specific resistance response on the cotton line AcBIn.

It is also possible that *pth*A encodes a pleiotropic virulence function needed for growth on bean. The simplest explanation, however, is that the avirulence phenotype of *pth*A is gratuitous at any host level, and does not limit host range on bean.

There has always been some question as to the role of the non-host HR in limiting host range, since HR is known to be a defense-associated response in plants, and the non-host HR is so general and common. Race-specificity is controlled by *avr* genes, which may limit the host range at higher than cultivar level, although usually within a plant family. For example, inactivation of *avr*BsT, a race-specificity gene of *X.c.* pv. *vesicatoria*, extends the host range of strains formerly able to attack only pepper to include tomato (Minsavage et al., 1990). The host range of *X.c.* pv. *vesicatoria* is well known to include both tomato and pepper (Bradbury, 1984), and presumably all cultivars of tomato tested so far carry the same *R* gene. Similarly, a *Pseudomonas solanacearum* AW1 *avr*A::Tn3-HoHo1 mutant strain was unable to cause an HR on tobacco plants and was able to grow at a faster rate *in planta*, unlike the parental wild type strain AW1 (Carney and Denny, 1990). These examples do not address the issue of whether *avr* genes in race-specificity on known hosts, not non-hosts. The HR *per se* is not a sufficient indicator of non-host status. In *X. citri*, elimination of the non-host HR in bean did not expand the host range beyond that already described. Work presented here supports the hypothesis that the host range (above race level) of biotrophic microbes may be determined by factors other than *avr* genes.

Gene *pth*A, in various *Xanthomonas* spp. is capable of eliciting an HR on non-hosts as well as canker-like lesions on citrus. Both phenotypes are essentially host

reponses to the presence of *pth*A in *Xanthomonas* strains. In the absence of *pth*A, such responses are not observed. It is possible that both the host responses may involve similar biochemical induction pathways in the plant. With *X. citri*, the citrus plant response that is induced may be beneficial for ecological spread and build-up of the pathogenic population; the non-host plant response that is induced on bean appears to be gratuitous.

CHAPTER IV
STRUCTURAL MANIPULATIONS OF *pth*A FROM *XANTHOMONAS CITRI* SEPARATE THE PLEIOTROPIC FUNCTIONS OF PATHOGENICITY AND AVIRULENCE

Introduction

Avirulence (*avr*) genes have been cloned from a number of plant pathogenic bacteria. Some *avr* genes have similarities to sequences found within the same strain or in other strains belonging to the same species. The genes *avr*B and *avr*C from *Pseudomonas syringae glycinea* (Staskawicz et al., 1987) are highly similar. Extensive homology exists between carboxy ends of the 50 kd and 100 kd proteins encoded by *avr*Bs1 of *Xanthomonas campestris vesicatoria* and *avr*A of *P.s. glycinea*, respectively (Keen, 1990). As a unique case, similarity has also been found between an *avr* gene (*avr*10) cloned from the pathogen *X.c. oryzae* and the gene *pho*S of *Escherichia coli* (Kelemu and Leach, 1990).

Recently, an *avr* gene family of *Xanthomonas* spp. was described (Defeyter and Gabriel, 1991). Members of the family are fairly widespread within the genus; a total of 25 of 38 strains tested belonging to 9 of 13 *Xanthomonas* pathovars contained sequences similar to these *avr* genes. Most of these sequences are plasmid borne and are present as multiple copies. Genes belonging to this family have unusually large sizes of 3-5 kb compared to typical prokaryotic genes. The large size is mainly due to the

presence of nearly identical 102 bp direct DNA repeats forming the core of the genes.

Most members of the *Xanthomonas avr* gene family were cloned by phenotypic expression of avirulence as evidenced by elicitation of hypersensitive response (HR) on resistant host cultivars. Examples are *avr*B2, *avr*B4, *avr*b6, *avr*b7, *avr*BIn, *avr*B101, and *avr*B102 of *X.c. malvacearum* (DeFeyter and Gabriel, 1991), and *avr*Bs3 (Bonas et al., 1989) and *avr*BsP of *X.c. vesicatoria* (Canteros and Stall, unpublished). One member of the family *pth*A, was cloned by phenotypic expression of pathogenicity as evidenced by elicitation of tissue hypertrophy leading to canker formation on citrus plants. This gene was later shown to determine the non-host HR of *X. citri* on bean, and also to elicit cultivar-specific avirulence in *X.c. malvacearum* on cotton. DNA hybridizations showed presence of sequences similar to all these *avr/pth* genes in *X. citri* and *X. phaseoli*. Neither species of *Xanthomonas* is known to display any race cultivar specificity on their hosts citrus and bean, respectively. I report here the cloning of all alleles belonging to the *Xanthomonas avr* gene family from *X. citri* and *X. phaseoli* and demonstrate that although none of these is known to function for avirulence on their hosts, they function as *avr* genes when assayed on cotton resistant lines in *X. c. malvacearum*. Furthermore, the pleiotropic functions of pathogenicity and avirulence of *pth*A could be separated by structural manipulations of its core region consisting of nearly identical DNA repeats.

Materials and Methods

Bacterial Strains, Plasmids and Bacteriological Techniques

All bacterial strains, growth conditions and culture media used in the study have been described in previous chapters. pZit45 carries *pth*A on a 4.5 kb DNA fragment in pUFR047, an 8.6 kb Gm^R Ap^R derivative of pUFR027 (DeFeyter et al., 1990). pZit45.119 is the subclone of the 4.5 kb insert from pZit45 cloned in pUC119 (Vieira and Messing, 1987). pCTR and pXPH contain genomic DNA fragments of *X. citri* 3213 and *X. phaseoli* G27 libraries in pUFR027 (DeFeyter et al., 1990) and pUFR043 (10.3 kb, Gm^R Nm^R) (DeFeyter and Gabriel, unpublished), respectively.

Bacteriological techniques involved in maintenance and transferring clones using triparental conjugations have been described previously.

Plant Growth Conditions and Pathogenicity Assays

The virulence enhancement pathogenicity assay involved screening transconjugants of *X.c. citrumelo* 3048 (an opportunistic leaf-spotting strain) carrying cloned DNA on citrus. Lesions characteristic of citrus canker disease caused by *X. citri* are large and erumpent, and have been described previously (Civerolo, 1984).

All the plants used for testing pathogenicity of *X. citri* 3213, its mutants or transconjugants were carried out in the quarantine greenhouse facilities at the Division of Plant Industry, Florida Department of Agriculture, Gainesville, FL. Inoculations

involving *X. citri* were carried out there under BL-3 level quarantine restrictions (refer Federal Register Vol. 52, No. 154, pp 29800-29814, August 11, 1987).

Citrus plants used were *Citrus paradisi* cv. Duncan (grapefruit). Bean plants were *Phaseolus vulgaris* cv. California Light Red (CLR) and cotton lines were *Gossypium hirsutum* cv. Acala 44 congenic resistant lines (DeFeyter and Gabriel, 1991).

Bacterial concentrations were adjusted to 10^8 cfu/ml inooculum and pressure infiltrated in the abaxial leaf surface as described previously. Observations on pathogenic symptoms were taken 5, 7, and 10 days after inoculation of citrus plants. Development of HR was observed regularly for 4 days on both bean and cotton plants.

Recombinant DNA Techniques and Structural Manipulations of *pth*A from *X. citri*

Standard recombinant DNA procedures for DNA extractions, analysis, hybridization and cloning were followed according to Sambrook et al. (1989).

pZit45 DNA was used to construct a series of deletion subclones. The strategy was based on the fact that the cloning vector of pZit45 (i.e., pUFR027) did not contain any *Bal*I site while single *Bal*I sires were present in all the repeats within *pth*A. pZit45 DNA was partially digested with *Bal*I and religated at (dilute) concentrations favoring formation of circular 'non-concatenated DNA. The resulting clones were analyzed by restriction analyses to determine the number of 102 bp DNA repeats deleted. A 123 bp DNA ladder (supplied by BRL) was used as a size marker. Two clones deleted for the same number of repeats were chosen randomly, thus providing a series of deletion

derivatives. This series containing 33 clones, was designated p45*Bal*IΔ. Only one clone p45*Bal*IΔ69 was recovered which had only one repeat deleted.

Results

Minimum Copies of *pth*A Related Sequences in *X. citri* 3213 and *X. phaseoli G27*

Both *Pst*I and *Sst*I recognition sites were previously shown to occur once within each member of the *Xanthomonas avr* gene family (Bonas et al., 1989; DeFeyter and Gabriel, 1991). *Eco*RI sites were not found in any of the genes characterized. We used combinations of these enzymes with *Eco*RI in single and double digests to generate restriction fragment length polymorphisms so as to determine the minimum number of *pth*A related sequences in *X. citri* 3213. Southern hybridization of *Eco*RI and *Sst*I double-digested *X. citri* 3213 DNA (Fig. 4-1) to radiolabelled *pth*A DNA (in pZit45.119) revealed at least four copies to be present in this strain. Similarly, *X. phaseoli* G27 contained at least two copies of sequences similar to *pth*A (data not shown).

Cloning of *pth*A Alleles from *X. citri* 3213 and *X. phaseoli* G27

Spot blots of genomic DNA library clones of *X. citri* 3213 and *X. phaseoli* G27 (in pUFRO43) (DeFeyter and Gabriel, unpublished) on nylon membranes were hybridized to radiolabelled *pth*A DNA (in pZit45.119). A total of 29 out of 576 clones tested from the *X. phaseoli*DNA library hybridized to the probe DNA, and 45 out of 1536 clones hybridized from the *X. citri* 3213 genomic DNA library. Clones obtained from the *X. citri* DNA library were designated pCTR 1 through 45 while those from the

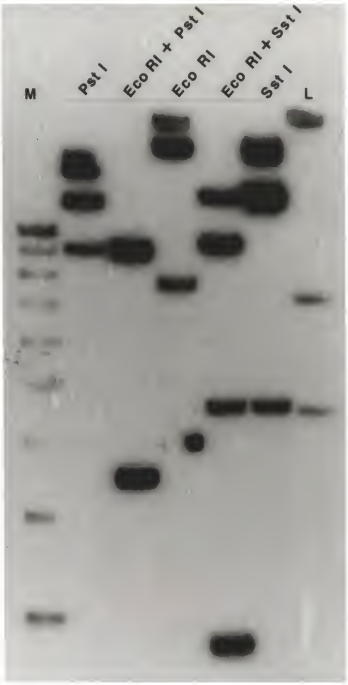


Fig. 4-1. Southern hybridization of *X. citri* 3213 digested genomic DNA with labelled *pth*A DNA. M, molecular size marker, 1 kb DNA leader; L, molecular size marker, HindIII digest of λ DNA.

X. phaseoli DNA library were designated pXPH 1 through 29. Based on results from hybridizations performed using a single copy gene as probe DNA, clones containing *pth*A related sequences were found to be over-represented in the genomic DNA libraries of both strains even after accounting for their multiple copies.

All 45 pCTR clones and 29 pXPH clones were digested with *Eco*RI and *Sst*I and hybridized to the radiolabelled *pth*A DNA probe (pZit45.119). Partial results from *X. citri* clones are shown in Fig. 4-2. Southern hybridization revealed that all four *pth*A related copies from *X. citri* 3213 and two copies from *X. phaseoli* G27 had been cloned in a number of pCTR and pXPH clones.

The bands corresponding to *pth*A related sequences in the ethidium bromide stained gels of genomic DNA digests of *X. citri* 3213 (shown by arrows in Fig. 4-2a) and *X. phaseoli* G27 (not shown) were found to be intense, suggesting multiple copies of each of these sequences. Taken together with the observation on overrepresentation of the sequences in the genomic DNA libraries, it strongly suggested that all these sequences may be plamid borne. Additionally, several pCTR and pXPH clones were found to contain more than one *pth*A allele (Fig. 4-2b) showing that several of these may be clustered on indigenous plasmids of *X. citri* 3213 and *X. phaseoli* G27.

Avirulence Functions of Cloned *pth*A Alleles in *X.c. malvacearum* on Cotton Congenic Resistant Lines

pCTR and pXPH clones containing various *pth*A alleles either singly or in combination were conjugally transferred to *X.c. malvacearum* N and the resulting transconjugants inoculated onto cotton cv. Acala 44 and its 9 congenic resistant lines.

Fig. 4-2. Southern hybridization of *Eco*RI + *Sst*I double digested DNA of pCTR clones with labelled *pth*A probe DNA. Lanes: M, molecular size marker -1 kb DNA ladder; 3213, *Eco*RI + *Sst*I digested genomic DNA of *X. citri* 3213. A. Ethidium bromide stained gel; B. Autoradiography resulting from Southern hybridization.

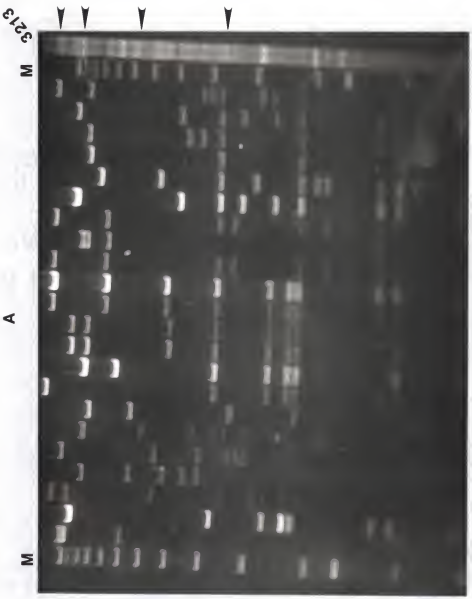

3213
M
A
M

Fig. 4-2 continued.

Table 4-1. Phenotype of *X.c. malvacearum* N containing *pth*A and its alleles from *X. citri* and *X. phaseoli* on ten cotton lines.

Host	Cotton cv. Acala 44 Congenic Resistant Lines									
Strain	Ac44	B1	B2	B4	B6	B4	BIn1	BIn3	20-3	82F2-1
X.c. malvacearum N	+	+	-	+	+	+	+	+	+	+
Xcm N/ *pth*A	+	-	-	+	+	+	-	-	-	-
Xcm N/ *avr*Xc1	+	+	-[w]	+	+	+	+	-[w]	+	-[w]
Xcm N/ *avr*Xc2	+	+	+	+	+	+	+	-	+	+
Xcm N/ *avr*Xc3	+	-	-	-	-	-	-	-	-	-
Xcm N/ *avr*Xp1	+	-	-	+	+	+	-	-	-	-

'+'= compatible interaction as evidenced by development of water-soaking symptoms; '−'=incompatible interactions as evidenced by development of hypersensitive response (HR); 'w'=intermediate HR development.

Besides the pleiotropic avirulence specificity of *pth*A reported previously, three new avirulence phenotypes were observed with pCTR clones in XcmN (Table 4-1). The alleles reponsible were designated *avr*Xc1, *avr*Xc2, and *avr*Xc3 (Table 4-1 and Fig. 4-2). XcmN containing *avr*Xc1 or *avr*Xc2 reacted in a cultivar-specific manner with cotton resistant lines. Xcm strain N with clones containing *avr*Xc3 elicited HR on cotton cv. Acala 44 and all resistant lines having Acala 44 genetic background.

Only one of the two *pth*A related sequences from *X. phaseoli* was found to encode avirulence when in XcmN (Table 4-1). The gene, designated *avr*Xp1, displayed the same cultivar-specific avirulence as *pth*A of *X. citri* on the cotton lines tested. The other allele may be a pseudogene or a pathogenicity gene without pleiotropic avirulence.

Phenotypes of Several Cloned *avr* Genes from *X.c. malvacearum* and *X. phaseoli* in *X. citri pth*A⁻ Mutant on Citrus cv. Duncan

Cloned *avr*B4, *avr*b6, *avr*7, *avr*BIn, *avr*B101and *avr*B102 of *X.c. malvacearum* and *avr*Xp1 from *X. phaseoli* were introduced into *X. citri* B21.2 (*pth*A::Tn5-Gus), a mutant without obvious symptoms on citrus. The transconjugants were assayed on citrus cv. Duncan (grapefruit) for complementation of the mutation by inoculating at 10^8 cfu/ml. Assay for complementation was the restoration of phenotype to pathogenic (ie., formation of cankerous lesions). In comparison to the degree of complementation by cloned *pth*A, the *avr* genes restored the phenotype only partially. A low level of pathogenic function was restored by introduction of *avr*BIn in strain B21.2, and an intermediate level of complementation was achieved by transconjugants harboring cloned *avr*Xp1, *avr*B4, *avr*b6, and *avr*B102. The presence of *avr*b7 and *avr*B101 did not affect

the phenotype of strain B21.2 in these assays. The phenotype conferred by *avr*Xp1 of *X. phaseoli* was also of the intermediate type when assayed in strain B21.2

Phenotypes conferred by *Bal*I deletion clones of *pth*A in *X. citri*B21.2 and *X.c. citrumelo* 3048 on citrus

The 33 *Bal*I deletion clones Table 4-2 were conjugally transferred into *X. citri* B21.2 and the resulting transconjugants were inoculated onto citrus cv. Duncan (grapefruit) at high (10^8 cfu/ml) bacterial concentrations. Of the 33 p45B*Bal*IΔ clones screened on citrus plants, 23 failed to elicit any response. Fifteen of these clones were deleted in more than 9 repeats. While intermediate pathogenic symptoms were elicited by B21.2/p45*Bal*IΔ clones 116 and 45, full pathogenicity (normosensitive response, NR) was restored by 5 *Bal*I deletion clones. Clones p45*Bal*IΔ 135, 53 and 26 deleted in 3, 4, and 8 repeats, respectively, elicited an HR on citrus (Table 4-2 and Fig. 4-3). The HR appeared 48 hours post-inoculation and lasted for another 48 hours before subsiding. Both the time of HR induction and its duration was similar to the HR elicited by the heterologous pathogen *X.c. malvacearum* under similar conditions.

The 33 p45*Bal*IΔ were evaluated by the 'virulence enhancement' assay used originally to clone *pth*A. Introduction of *pth*A into *X.c. citrumelo* 3048 leads to formation of cankers on citrus. Three clones which complemented the pathogenicity function of strain B21.2 also enabled *X.c. citrumelo* strain 3048 to induce cankers on citrus. However, no p45*Bal*IΔ clone was found to have an avirulence activity in strain 3048 on citrus.

Table 4-2. Phenotypes of *X. citri* B21.2 (*pth*A) and *X.c. citrumelo* 3048 containing p45*Bal*IΔ clones on citrus cv. Duncan (grapefruit). Path$^+$ = development of canker-like lesions; Path$^+$(I) = partial development of canker-like lesions; 'o' = null response; '+' = indicates development of leaf spot symptoms.

No. of Repeats Deleted	p45B*al*IΔ.#	Grapefruit cv. Duncan	
		B21.2/B*al*I Δ	3048/B*al*IΔ
0	--	Path^{+} [1]	Path^{+}
1	69	0	+
2	79	0	+
	122	0	+
4	44	Path^{+}	Path^{+}
	135	HR	+
4	53	HR	+
	79	Path^{+}	Path^{+}
5	39	0	+
	117	0	+
6	107	0	+
	123	Path^{+}	Path^{+}
7	116	Path^{+}(I)	Path^{+}
	144	Path^{+}	Path^{+}
8	26	HR	+
	62	0	+
9	47	0	+
	67	Path^{+}	Path^{+}(I)
10	77	0	+
	140	0	+
11	68	0	+
	104	0	+
12	72	0	+
	114	0	+
13	27	0	+
	101	0	+
14	45	Path^{+}(I)	Path^{+}(I)
	56	0	+
15	20	0	+
	94	0	+
16	23	0	+
	71	0	+
17	18	0	+
	81	0	+

Fig. 4-3. Phenotypes of *X. citri* B21.2 (*pth*A⁻) containing p45 *Bal*IΔ, clones on a citrus cv. Duncan leaf. A. Full restoration of of pathogenicity by B21.2/p45*Bal*IΔ123; B. Partial complementation of pathogenicity by B21.2/p45*Bal*IΔ45; C. Hypersensitive response induced by B21.2/p45*Bal*IΔ26; D. Null response induced by B21.2/p45*Bal*IΔ47.

Avirulence Activity of *pth*A *Bal*I Deletion Clones in *X. citri* B21.2 and *X.c. citrumelo* 3048 on Bean cv. CLR

The ability to induce non-host HR by *X. citri* 3213 on bean cv. CLR is lost in the mutant strain B21.2 (*pth*A::Tn5-Gus). The set of 33 *Bal*I deletion clones of *pth*A in B21.2 were assayed for their ability to complement the loss of function (ie., lack of HR inducing ability) on bean plants. Introduction of *pth*A into *X.c. citrumelo* 3048, a mild pathogen of bean, renders it avirulent on cultivar CLR. The p45*Bal*IΔ clones were introduced into strain 3048 and assayed on bean plants for elicitation of HR. Seven clones conferred avirulence to both the strains (ie., B21.2 and 3048). These avirulent induced an HR on bean leaves to the same extent as that induced by the corresponding strains carrying unmodified *pth*A (Fig. 4-4). All clones with intact avirulence function had between 8 and 13 repeats deleted. Among the rest, an almost equal number of clones had lost all or some avirulence activity, based on a visual assessment of the strength of the HR. Nine of the 13 tested clones containing less than 8 repeats deleted had lost all avirulence activity

Avirulence of *X.c. malvacearum* N Containing p45*Bal*IΔ's on Five Congenic Cotton Resistant Lines

Transconjugants of XcmN containing p45*Bal*IΔ clones were inoculated at 10^8 cfu/ml inoculum on five congenic cotton resistant lines (Table 4-3). Two cotton lines (viz., Acala 44 and B7) selected were susceptible to XcmN/pZit45 and three others (B1, B5-22, and BIn3) were resistant. Avirulence specificity conferred by six p45*Bal*IΔ clones was found to be the same as that of pZit45. Twenty seven of the N/p45*Bal*IΔ clones

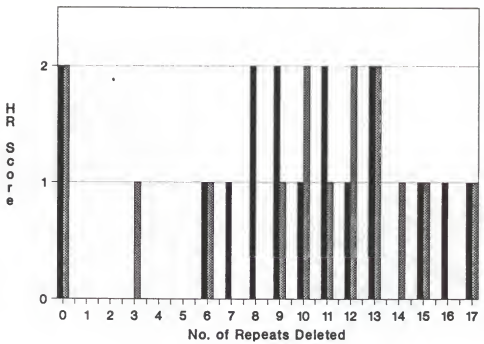


Fig 4-4. HR score of 33 p45*BalI*Δ clones in B21.2 (or 3048) on bean cv. CLR. The two bar fill patterns represent the phenotypes conferred by two clones carrying the same sized deletion HR Score 0= No HR induced; 1= Intermediate level of HR; 2= Full HR. All scores based on relative intensities of HR compared to that induced by *pth*A.

Table 4-3. Phenotypes of *X.c. malvacearum* N containing p45*Bal*IΔ clones on 5 cotton lines. '+'= compatible interaction as evidenced by development of water-soaking symptoms; '-'= incompatible interaction as evidenced by development of a hypersensitive response (HR); 'w'= intermediate HR development; +'= increased virulence as indicated by increased water-soaking symptoms.

No. of Repeats Deleted	p45*Ball*Δ#	*X.c. malvacearum* N				
		Ac44	AcB1	AcB5	Acb7	AcBIn3
0	–	+	-	-	+	-
0	69	-	+	+	+	+
7	79	+	-[w]	+	+	-
	122	+	+	-[w]	+	+
7	44	+	+	-	+	+
	101	+	-	-	+	-
6	69	-	-[w]	-	+	-
	79	-	+	-	+	-
5	79	-[w]	-	-	+	+
	114	+	+	-[w]	+	+
6	101	+	-	-	+	-[w]
	123	+	-	-	+	+
7	101	+	+	-[w]	+	+
	144	-[w]	+	-[w]	+	+
8	26	+	-	-	-	-
	69	+	-	-	+	-
9	47	-	-[w]	-	-[w]	-[w]
	47	+	-	-	+	+
10	77	+	-	-	-	-
	140	+	-	-	+	-
13	69	-[w]	-	-	+	-
	104	+	-	-	+	-
12	72	+	-	-	-	+
	114	-[w]	-	-	+	-
13	27	-	-	-	-	-
	101	-	-	-	+	-
13	45	+	+*	+*	+*	+*
	56	+	-	-	+	-
10	60	+	-	-	-	+
	69	+	-	-	-	-
16	23	+	-	-	-	-
	45	+	-	-	-	-
17	18	+	-	-	-	-
	81	+	-	-	-	-

exhibited avirulence but altered specificity from that of N/pZit45. Of these, 7 were avirulent on all 4 resistant lines tested and 2 were additionally avirulent on Acala 44 as. Strain N/p45*Ball*Δ45 was found to have increased the water soaking ability of strain N on all cotton lines tested.

Discussion

Avirulence genes from a number of plant pathogenic bacteria have been characterized but only a few seem to be similar either to each other or to sequences with any described functions (Keen, 1990). The *Xanthomonas avr* gene family (DeFeyter and Gabriel, 1991) seems by far to be the most widespread, and a relatively large number of these have been at least partially characterized. Six *avr* genes from *X.c. malvacearum* (De Feyter and Gabriel, 1991) and two from *X.c. vesicatoria* (Bonas et al., 1989; Canteros and Stall, unpublished) along with one pathogenicity gene from *X. citri* have been shown to belong to this family. The members of the family are predominantly plasmid borne, and unusually large sized (3-5 kb) for average prokaryotic genes (DeFeyter and Gabriel, 1991).

We report here the cloning of three *pth*A related sequences from *X. ciri* and two from *X. phaseoli*, and their avirulence function on heterologous hosts. One *pth*A related sequence in *X. phaseoli* was found to be non-functional as an *avr* gene in our assays. The non-host avirulence was assayed in *X.c. citrumelo* on bean and in *X.c. malvacearum* N inoculated on cotton cv. Acala 44 and its nine congenic resistant lines. All four *avr* genes possessed unique specificities for interaction. Those of *avr* Xc1 and *avr*Xc2 were

quite similar (Table 4-1). However, the two alleles were physically distinguishable by hybridization of genomic digest to labelled *pth*A DNA probe (Fig. 4-2). Gene *avr*Xc3, seemed to recognize an as yet unknown resistance gene in Acala 44 so that it reacted on all the ten cotton lines used in this study. Marker exchanged *pth*A in *X. citri pth*A$^-$ strain B21.2 was shown previously to be avirulent an all ten cotton lines tested, and *avr*Xc3 could be solely responsible for this heterologous reaction. Gene *pth*A has been previously shown to be responsible for the non-host HR on bean cv. CLR. Gene *avr*Xp1, from *X. phaseoli* possessed avirulence activity as detected in our assays. The avirulence specificity (Table 4-1) as well as the size of the gene (not shown) was the same as that of *pth*A of *X. citri* on the cotton lines tested. Since *X. phaseoli* elicits a non-host HR on Acala 44, presumably other *avr* genes are involved in this reaction.

Based on the fact that all *X.c. malvacearum* and *X. phaseoli* cloned *avr* gene were highly similar to *pth*A of *X. citri*, we investigated whether some of them would be able to complement the lack of pathogenicity of *X. citri* B21.2 (*pth*A$^-$) on citrus. Four *avr* genes from *X.c. malvacearum* and one from *X. phaseoli* partially restored the pathogenicity function of *pth*A in the non-pathogenic *X. citri* mutant B21.2. Unlike *pth*A, none of these *avr* genes were found to enhance the virulence of *X.c. citrumelo* 3048 on citrus. Therefore, gene *pth*A may be more adapted for pathogenicity function on citrus than the other *Xanthomonas avr* genes tested.

Members of the *Xanthomonas avr* gene family have a peculiar structure. The core region consists of multiple, nearly identical 102 bp direct DNA repeats (Bonas et al., 1989). All the members with different avirulence specificities vary in the number

of these repeats (DeFeyter and Gabriel, 1991). The presence of the repeats also accounts for the unusually large size of the genes. I investigated whether manipulation in the core region of gene *pth*A could affect its pleiotropic biological functions. Presence of a *Bal*I site within each repeat unit and nowhere else in the gene or the cloning vector allowed the construction of a set of deletion clones which had various sets of repeats in the core region while leaving the rest of gene intact. Since each repeat is 102 bp in size, deletions of complete repeats would not change the reading frame. However, the *Bal*I sites are 20 bp from the start of each repeat and this results in the creation in each *Bal*I deletion clone of a chimeric repeat not originally present in *pth*A.

A set of *Bal*I deletion clones of *pth*A (p45*Bal*IΔs; Δ1, Δ2, Δ3...Δ17) were chosen such that two independent deletion clones were selected that had randomly lost the same number of repeats. The intended purpose was to study whether the number of repeats, specific repeats, or both, play a role in the pleiotropic biological functions. Effects of deletions of repeats on pathogenicity were studied by ability of clones to complement the loss of function in *X. citri* B21.2 and by their ability to enhance pathogenicity of *X.c. citrumelo* 3048. Most of the deletion clones were found to have lost the pathogenicity function. The same clones which imparted pathogenicity to B21.2 also enhanced the virulence of *X.c. citrumelo* 3048. It was significant to note that three deletion clones conferred ability of B21.2 to elicit an HR on citrus (Fig. 4-3). The HR on citrus has not previously been described.

On bean, the loss of ability of *X. citri* B21.2 to induce HR was restored partially by 14 deletion clones and fully by 7 deletion clones (Fig. 4-4). These same clones also

rendered *X.c. citrumelo* 3048 incompatible, indicating that the basis of HR induction by *X. citri* and *X. c. citrumelo* on bean is quite similar. All clones in B21.2 and 3048 that induced an HR on bean plants had lost in their pathogenicity function. In general, clones containing between 8 to 13 repeats deleted still retained the ability to function for this avirulence.

On cotton, similar results were observed in terms of non-host HR. As with HR induction on bean, clones in Xcm with larger deletions (more than 14 repeats deleted) were still avirulent. However, a loss of cultivar-specificity was observed on the 5 cotton lines tested.

p45*Bal*IΔ47 in *X.c. malvacearum* N elicited an HR not only on all cotton lines (including Acala 44) but it also had an avirulence activity in *X. citri* B21.2 and *X.c. citrumelo* 3048 on bean cv. CLR. It had, therefore, lost the specificity, yet retained and widened the avirulence function. On the other hand, p45*Bal*IΔ45 was found to have lost both avirulence and specificity but gained a non-specific virulence function on citrus and all cotton lines. Unlike *pth*A, which does not increase water soaking of XcmN on cotton, (a normosensitive, or NR response), p45*Bal*I 45 not only enhanced virulence of strain N on cotton, but also enhanced virulence of strain 3048 on citrus and fully complemented the pathogenicity of strain B21.2. By manipulation of *pth*A in the DNA repeat containing core region, therefore, not only could pathogenicity be separated from avirulence, but also enhanced pathogenicity for two hosts simultaneously could also be achieved. Presumably the relatively large number of copies of alleles of this *avr* gene family typical for any given *Xanthomonas* strain would provide a mechanism to generate

variation in these genes which could then be naturally selected. The very fact that so many functional copies of these genes are found in so many *Xanthomonas* strains is evidence that there is survival value in preserving functioning copies of these genes. The fact that the pleiotropic avirulence functions may be separated from the pathogenicity function is an indication that the avirulence function is unnecessary and possibly gratuitous.

CHAPTER V
SUMMARY AND CONCLUSIONS

A virulence enhancement approach was used to clone a pathogenicity (*pth*) locus from a highly virulent pathogen by assaying the library in a second, less virulent strain that was compatible with the same host. A genomic library of the virulent Asiatic canker pathogen *Xanthomonas citri* was conjugally transferred to the opportunistic pathogen, *X. campestris* pv. *citrumelo*, and the transconjugants were screened on *Citrus paradisi* cv. "Duncan" (grapefruit) leaves. Transconjugants able to induce host cell proliferation and raised, Asiatic canker-like lesions were identified, and clone pSS10.35 was found to carry the gene(s) responsible. This clone was transferred to other *Xanthomonas* strains, including two that are weakly pathogenic to citrus in greenhouse tests (members of *X.c.* pvs. *alfalfae* and *cyamopsidis*), and two that are avirulent on citrus (*X. phaseoli* and *X.c.* pv. *malvacearum*). Transconjugants of the two weakly pathogenic *Xanthomonas* strains induced canker-like lesions when inoculated on citrus; these same strains became avirulent on their homologous host plants. Transconjugants of *X. phaseoli* and *X.c. malvacearum* strains remained unaltered in phenotype on citrus. A 3.7 kb region of pSS10.35 carrying the *pth*A locus was identified by subcloning and Tn*5-Gus* mutagenesis. Marker-exchange mutagenesis of *X. citri* using Tn*5-Gus* insertions in the 3.7 kb region resulted in a complete loss of virulence (disease symptoms and growth *in planta*) on citrus and loss of the hypersensitive response on heterologous hosts (i.e., an

Hrp$^-$ phenotype). The Hrp$^-$ phenotype, but not growth *in planta*, of the marker-exchanged mutants was restored by subclones of pSS10.35 containing the 3.7 kb region.

When introduced into strains of *X. phaseoli*, *X. campestris* pv. *cyamopsidis*, and *X.c.* pv. *malvacearum*, (none pathogenic to citrus), the transconjugants remained nonpathogenic to citrus and elicited a hypersensitive response (HR) on their respective hosts bean, guar, and cotton. In *X.c.* pv. *malvacearum*, *pth*A behaved as a classical cultivar-specific avirulence (*avr*) gene on congenic cotton resistant lines. Structurally, *pth*A is similar to *avr*Bs3 from *X.c.* pv. *vesicatoria* and to *avr*B4, *avr*b6, *avr*b7, *avr*BIn, *avr*B101 and *avr*B102 from *X.c.* pv. *malvacearum*. Marker-exchanged *pth*A::Tn*5*-Gus mutations of *X. citri* were nonpathognenic to citrus and no longer induced the heterologous (non-host) HR when inoculated on bean cv. California Light Red. The heterologous HR was induced by both *X. citri* wild type and *pth*A::Tn5-Gus mutants when inoculated onto cotton. The loss of the ability of *X. citri* marker-exchanged mutants to elicit HR on bean was restored by introduction of cloned *pth*A, indicating that the genetics of the heterologous HR may be the same as that of homologous HR and involving specific *avr* genes, such as *pth*A. In contrast with expectations of homologous HR reactions, however, elimination of *pth*A function (resulting in loss of HR) did not result in water-soaking or increased growth *in planta* of *X. citri* on bean; the heterologous HR is, therefore, not responsible for resistance of bean to *X. citri* and does not limit the host range of *X. citri* on bean. The pleiotropic avirulence function of *pth*A and the heterologous HR of bean to *X. citri* are both evidently gratuitous.

Three genes from *X. citri* 3213 in addition to *pth*A *viz.*, *avr*Xc1, *avr*Xc2, and *avr*Xc3 were identified by hybridization and cloned. All members of the *avr* gene family from *X. citri* exhibited cultivar-specific avirulence in *X.c. malvacearum* N on cotton resistant lines even though no race-cultivar interactions are known between *X. citri* and its rutaceous hosts. These genes appeared not to be involved in virulence of *X. citri* on citrus (Grapefruit cv. Duncan). This was also evidenced by the inability of the *avr* genes either to complement *pth*A mutation in *X. citri* B21.2 or to enhance virulence of the mild pathogen *X.c. citrumelo* 3048 when assayed on citrus. Gene *pth*A and its alleles had 102 bp tandemly arranged, nearly identical direct repeated DNA in their core region which accounted for their size variation. Variously sized deletions of the repeats were constructed and the clones assayed for pathogenicity on citrus (in *X. citri* B21.2 and *X.c. citrumelo* 3048) and avirulence on bean (in B21.2 and 3048) and cotton resistant lines (in *X.c. malvacearum* N). A number of clones with large number of repeats deleted (i.e., more than 10 of the 17 present in *pth*A) conferred non-specific avirulence to *X.c. malvacearum* on all four cotton resistant lines tested. Pathogenicity on citrus was restored either fully or partially by eight of the 33 deletion derivatives assayed in *X. citri* B21.2. Several clones were identified which had separated the gratuitous pleiotropic function of avirulence from pathogenicity. One clone (deleted in nine repeats) had lost the pathogenicity function but gained exaggerated avirulence and conferred non-specific avirulence on all hosts and all lines of cotton studied; another clone (deleted in 14 repeats) had completely lost avirulence activity but gained ability to enhance pathogenicity of *X.c. malvacearum* on all cotton lines and *X.c. citrumelo* on citrus in

addition to complementing pathogenicity of B21.2 on citrus. Surprisingly, three deletion derivatives of *pth*A in *X. citri* B21.2 induced hypersensitive response in citrus, whereas these clones in *X.c. citrumelo* 3048 (which lacks *pth*A related sequences) had no activity on the same host. This suggested an involvement of other gene(s) in *X. citri* which may be required for avirulence activity on citrus.

It has been suggested previously that normosensitive (NR) and hypersensitive responses (HR) may be quite similar in nature. In the present study, it was found that simple *in vitro* manipulations were able to convert one type of response elicitation to another strongly reiterating similar nature of NR and HR elicitation. The observation that several clones separated the two functions indicated that although normosensitive and hypersensitive responses may be quite similar it would be possible for a number of naturally occurring genes to possess either of these functions. *avr* genes which would possess the ability to elicit normosensitive response would then possess a selective value for the pathogen which may lead to their widespread distribution. Evidence was obtained that this may be the case with at least *pth*A of the *Xanthomonas avr* gene family.

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The author was born on June 23, 1961 to Suman and Vishnu Swarup in Lucknow, Uttar Pradesh, in India. He obtained his primary and secondary school education from the Modern High School in New Delhi. To pursue a career in agriculture the author went to the Punjab Agricultural University, Ludhiana, Punjab, India for his B.Sc. (Ag.) Hons. degree which he obtained in 1983. He did his M.Sc. and Ph.D in genetics with a major in plant breeding and minors in biochemistry and molecular biology at the Indian Agricultural Research Institute, New Delhi. Soon after completing his degree and research requirements for the Ph.D., Sanjay Swarup got an opportunity to work with Dr. Dean Gabriel at University of Florida, Gainesville, Florida. In the summer of 1990, the author went back to India to defend his Ph.D. dissertation and graduated in February, 1991 from I.A.R.I., New Delhi.

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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